

# INTERNATIONAL SEARCH REPORT

International Application No

PCT/FR 99/02995

## A. CLASSIFICATION OF SUBJECT MATTER

IPC 7 C12N15/87 A61K48/00 C07H15/04

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

MEDLINE, BIOSIS, CHEM ABS Data

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	<p>FUHRHOP J -H ET AL: "BOLAAMPHIPHILES WITH MANNOSE- AND TETRAALKYLAMMONIUM HEAD GROUPS ASCOATINGS FOR NUCLEIC ACIDS AND POSSIBLE REAGENTS FOR TRANSFECTIONS" CHEMISTRY AND PHYSICS OF LIPIDS, vol. 43, 1 April 1987 (1987-04-01), pages 193-213, XP000562618 ISSN: 0009-3084</p>	1-5, 8-12, 18-22, 26,28-33
Y	<p>the whole document</p>	23-25,27
Y	<p>WO 96 25508 A (RHONE POULENC RORER SA ;BYK GERARDO (FR); SCHERMAN DANIEL (FR); SC) 22 August 1996 (1996-08-22) cited in the application the whole document</p>	23-25,27

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

\* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search

30 June 2000

Date of mailing of the international search report

06/07/2000

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2  
NL - 2280 HV Rijswijk  
Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,  
Fax: (+31-70) 340-3016

Authorized officer

Sitch, W

# INTERNATIONAL SEARCH REPORT

Intern. Application No

PCT/FR 99/02995

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	<p>WO 98 06869 A (PHILADELPHIA CHILDREN HOSPITAL) 19 February 1998 (1998-02-19)  page 5, line 26 -page 10, line 30  page 16, line 7 -page 35, line 15</p> <p style="text-align: center;">---</p>	
A	<p>WO 96 21036 A (VIAGENE INC)  11 July 1996 (1996-07-11)  page 2, line 13 -page 3, line 29  page 5, line 8 - line 16</p> <p style="text-align: center;">---</p>	
A	<p>DATABASE CHEMICAL ABSTRACTS 'Online!  abstract no. 126:207866,  ERBACHER ET AL: "THE REDUCTION OF THE  POSITIVE CHARGES OF POLYLYSINE BY PARTIAL  GLUCONOYLATION INCREASES THE TRANSFECTION  EFFICIENCY OF POLYLYSINE/DNA COMPLEXES"  XP002116398  abstract  &amp; BIOCHIM. BIOPHYS. ACTA,  vol. 1324, no. 1, 1997, pages 27-36,</p> <p style="text-align: center;">---</p>	
A	<p>DATABASE MEDLINE 'Online!  US NATIONAL LIBRARY OF MEDICINE (NLM),  BETHESDA, MD, US;  abstract 1999129193,  GOULA ET AL: "POLYETHYLENIMINE-BASED  INTRAVENOUS DELIVERY OF TRANSGENES TO  MOUSE LUNG"  XP002116399  abstract  &amp; GENE THERAPY,  vol. 5, no. 9, September 1998 (1998-09),  pages 1291-1295,</p> <p style="text-align: center;">---</p>	
A	<p>DATABASE MEDLINE 'Online!  US NATIONAL LIBRARY OF MEDICINE (NLM),  BETHESDA, MD, US;  ABSTRACT 96200295,  OKU ET AL: "EFFECT OF SERUM PROTEIN  BINDING ON REAL-TIME TRAFFICKING OF  LIPOSOMES WITH DIFFERENT CHARGES ANALYZED  BY POSITRON EMISSION TOMOGRAPHY"  XP002116400  abstract  &amp; BIOCHIMICA ET BIOPHYSICA ACTA,  vol. 1280, no. 1,  3 April 1996 (1996-04-03), pages 149-154,</p> <p style="text-align: center;">-----</p>	



# INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/FR 99/02995

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9625508 A	22-08-1996	FR 2730637 A	23-08-1996
		AU 706643 B	17-06-1999
		AU 4835396 A	04-09-1996
		BR 9607383 A	25-11-1997
		CA 2211162 A	22-08-1996
		CZ 9702592 A	12-11-1997
		EP 0809705 A	03-12-1997
		FI 973363 A	15-08-1997
		HU 9801207 A	28-08-1998
		JP 11500431 T	12-01-1999
		NO 973745 A	14-08-1997
		SK 111897 A	04-02-1998
		US 5945400 A	31-08-1999
		ZA 9601255 A	27-08-1996
WO 9806869 A	19-02-1998	US 5948681 A	07-09-1999
		AU 4065997 A	06-03-1998
WO 9621036 A	11-07-1996	AU 4690596 A	24-07-1996



2

1

2

4

# RAPPORT DE RECHERCHE INTERNATIONALE

Dem. : Internationale No

PCT/FR 99/02995

**A. CLASSEMENT DE L'OBJET DE LA DEMANDE**  
CIB 7 C12N15/87 A61K48/00 C07H15/04

Selon la classification internationale des brevets (CIB) ou à la fois selon la classification nationale et la CIB

**B. DOMAINES SUR LESQUELS LA RECHERCHE A PORTE**

Documentation minimale consultée (système de classification suivi des symboles de classement)

CIB 7 A61K

Documentation consultée autre que la documentation minimale dans la mesure où ces documents relèvent des domaines sur lesquels a porté la recherche

Base de données électronique consultée au cours de la recherche internationale (nom de la base de données, et si réalisable, termes de recherche utilisés)

MEDLINE, BIOSIS, CHEM ABS Data

**C. DOCUMENTS CONSIDERES COMME PERTINENTS**

Catégorie *	Identification des documents cités, avec, le cas échéant, l'indication des passages pertinents	no. des revendications visées
X	FUHRHOP J -H ET AL: "BOLAAMPHIPHILES WITH MANNOSYL AND TETRAALKYLAMMONIUM HEAD GROUPS AS COATINGS FOR NUCLEIC ACIDS AND POSSIBLE REAGENTS FOR TRANSFECTIONS" CHEMISTRY AND PHYSICS OF LIPIDS, vol. 43, 1 avril 1987 (1987-04-01), pages 193-213, XP000562618 ISSN: 0009-3084	1-5, 8-12, 18-22, 26,28-33
Y	le document en entier	23-25,27
Y	WO 96 25508 A (RHONE-POULENC RORER SA ; BYK GERARDO (FR); SCHERMAN DANIEL (FR); SC) 22 août 1996 (1996-08-22) cité dans la demande le document en entier	23-25,27
	-/-	

☒ Voir la suite du cadre C pour la fin de la liste des documents

☒ Les documents de familles de brevets sont indiqués en annexe

\* Catégories spéciales de documents cités:

"A" document définissant l'état général de la technique, non considéré comme particulièrement pertinent

"E" document antérieur, mais publié à la date de dépôt international ou après cette date

"L" document pouvant jeter un doute sur une revendication de priorité ou cité pour déterminer la date de publication d'une autre citation ou pour une raison spéciale (telle qu'indiquée)

"O" document se référant à une divulgation orale, à un usage, à une exposition ou tous autres moyens

"P" document publié avant la date de dépôt international, mais postérieurement à la date de priorité revendiquée

"T" document ultérieur publié après la date de dépôt international ou la date de priorité et n'appartenant pas à l'état de la technique pertinent, mais cité pour comprendre le principe ou la théorie constituant la base de l'invention

"X" document particulièrement pertinent; l'invention revendiquée ne peut être considérée comme nouvelle ou comme impliquant une activité inventive par rapport au document considéré isolément

"Y" document particulièrement pertinent; l'invention revendiquée ne peut être considérée comme impliquant une activité inventive lorsque le document est associé à un ou plusieurs autres documents de même nature, cette combinaison étant évidente pour une personne du métier

"&" document qui fait partie de la même famille de brevets

Date à laquelle la recherche internationale a été effectivement achevée

30 juin 2000

Date d'expédition du présent rapport de recherche internationale

06/07/2000

Nom et adresse postale de l'administration chargée de la recherche internationale  
Office Européen des Brevets, P.B. 5818 Patentiaan 2  
NL - 2280 HV Rijswijk  
Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,  
Fax: (+31-70) 340-3016

Fonctionnaire autorisé

Sitch, W

# RAPPORT DE RECHERCHE INTERNATIONALE

Dem: Internationale No  
PCT/FR 99/02995

C.(suite) DOCUMENTS CONSIDERES COMME PERTINENTS		
Catégorie	Identification des documents cités, avec, le cas échéant, l'indication des passages pertinents	no. des revendications visées
A	<p>WO 98 06869 A (PHILADELPHIA CHILDREN HOSPITAL) 19 février 1998 (1998-02-19) page 5, ligne 26 -page 10, ligne 30 page 16, ligne 7 -page 35, ligne 15</p> <p>---</p>	
A	<p>WO 96 21036 A (VIAGENE INC) 11 juillet 1996 (1996-07-11) page 2, ligne 13 -page 3, ligne 29 page 5, ligne 8 - ligne 16</p> <p>---</p>	
A	<p>DATABASE CHEMICAL ABSTRACTS 'en ligne! abstract no. 126:207866, ERBACHER ET AL: "THE REDUCTION OF THE POSITIVE CHARGES OF POLYLYSINE BY PARTIAL GLUCONOYLATION INCREASES THE TRANSFECTION EFFICIENCY OF POLYLYSINE/DNA COMPLEXES" XP002116398 abrégé &amp; BIOCHIM. BIOPHYS. ACTA, vol. 1324, no. 1, 1997, pages 27-36,</p> <p>---</p>	
A	<p>DATABASE MEDLINE 'en ligne! US NATIONAL LIBRARY OF MEDICINE (NLM), BETHESDA, MD, US; abstract 1999129193, GOULA ET AL: "POLYETHYLENIMINE-BASED INTRAVENOUS DELIVERY OF TRANSGENES TO MOUSE LUNG" XP002116399 abrégé &amp; GENE THERAPY, vol. 5, no. 9, septembre 1998 (1998-09), pages 1291-1295,</p> <p>---</p>	
A	<p>DATABASE MEDLINE 'en ligne! US NATIONAL LIBRARY OF MEDICINE (NLM), BETHESDA, MD, US; ABSTRACT 96200295, OKU ET AL: "EFFECT OF SERUM PROTEIN BINDING ON REAL-TIME TRAFFICKING OF LIPOSOMES WITH DIFFERENT CHARGES ANALYZED BY POSITRON EMISSION TOMOGRAPHY" XP002116400 abrégé &amp; BIOCHIMICA ET BIOPHYSICA ACTA, vol. 1280, no. 1, 3 avril 1996 (1996-04-03), pages 149-154,</p> <p>-----</p>	

# RAPPORT DE RECHERCHE INTERNATIONALE

Renseignements relatifs aux membres de familles de brevets

Demande Internationale No

PCT/FR 99/02995

Document brevet cité au rapport de recherche	Date de publication	Membre(s) de la famille de brevet(s)	Date de publication
W0 9625508 A	22-08-1996	FR 2730637 A	23-08-1996
		AU 706643 B	17-06-1999
		AU 4835396 A	04-09-1996
		BR 9607383 A	25-11-1997
		CA 2211162 A	22-08-1996
		CZ 9702592 A	12-11-1997
		EP 0809705 A	03-12-1997
		FI 973363 A	15-08-1997
		HU 9801207 A	28-08-1998
		JP 11500431 T	12-01-1999
		NO 973745 A	14-08-1997
		SK 111897 A	04-02-1998
		US 5945400 A	31-08-1999
		ZA 9601255 A	27-08-1996
W0 9806869 A	19-02-1998	US 5948681 A	07-09-1999
		AU 4065997 A	06-03-1998
W0 9621036 A	11-07-1996	AU 4690596 A	24-07-1996



...

...

3.

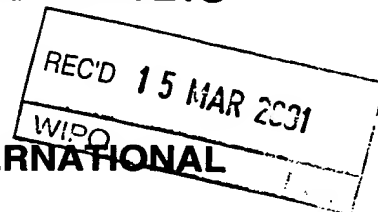
...



# TRAITE DE COOPERATION EN MATIERE DE BREVETS

## PCT

### RAPPORT D'EXAMEN PRELIMINAIRE INTERNATIONAL

(article 36 et règle 70 du PCT)



Référence du dossier du déposant ou du mandataire ST98046	<b>POUR SUITE A DONNER</b> voir la notification de transmission du rapport d'examen préliminaire international (formulaire PCT/IPEA/416)	
Demande internationale n° PCT/FR99/02995	Date du dépôt international (jour/mois/année) 02/12/1999	Date de priorité (jour/mois/année) 03/12/1998
Classification internationale des brevets (CIB) ou à la fois classification nationale et CIB C12N15/87		
Déposant AVENTIS PHARMA S.A		
<p>1. Le présent rapport d'examen préliminaire international, établi par l'administration chargée de l'examen préliminaire international, est transmis au déposant conformément à l'article 36.</p> <p>2. Ce RAPPORT comprend 7 feuilles, y compris la présente feuille de couverture.</p> <p><input type="checkbox"/> Il est accompagné d'ANNEXES, c'est-à-dire de feuilles de la description, des revendications ou des dessins qui ont été modifiées et qui servent de base au présent rapport ou de feuilles contenant des rectifications faites auprès de l'administration chargée de l'examen préliminaire international (voir la règle 70.16 et l'instruction 607 des Instructions administratives du PCT).</p> <p>Ces annexes comprennent feuilles.</p>		
<p>3. Le présent rapport contient des indications relatives aux points suivants:</p> <ul style="list-style-type: none"> <li>I <input checked="" type="checkbox"/> Base du rapport</li> <li>II <input type="checkbox"/> Priorité</li> <li>III <input checked="" type="checkbox"/> Absence de formulation d'opinion quant à la nouveauté, l'activité inventive et la possibilité d'application industrielle</li> <li>IV <input type="checkbox"/> Absence d'unité de l'invention</li> <li>V <input checked="" type="checkbox"/> Déclaration motivée selon l'article 35(2) quant à la nouveauté, l'activité inventive et la possibilité d'application industrielle; citations et explications à l'appui de cette déclaration</li> <li>VI <input type="checkbox"/> Certains documents cités</li> <li>VII <input checked="" type="checkbox"/> Irrégularités dans la demande internationale</li> <li>VIII <input type="checkbox"/> Observations relatives à la demande internationale</li> </ul>		
Date de présentation de la demande d'examen préliminaire internationale 07/06/2000	Date d'achèvement du présent rapport 12.03.2001	
Nom et adresse postale de l'administration chargée de l'examen préliminaire international:  Office européen des brevets D-80298 Munich Tél. +49 89 2399 - 0 Tx: 523656 epmu d Fax: +49 89 2399 - 4465	Fonctionnaire autorisé  G. Willière  N° de téléphone +49 89 2399 8548 	





**RAPPORT D'EXAMEN  
PRÉLIMINAIRE INTERNATIONAL**

Demande internationale n° PCT/FR99/02995

**I. Base du rapport**

1. Ce rapport a été rédigé sur la base des éléments ci-après (*les feuilles de remplacement qui ont été remises à l'office récepteur en réponse à une invitation faite conformément à l'article 14 sont considérées dans le présent rapport comme "initialement déposées" et ne sont pas jointes en annexe au rapport puisqu'elles ne contiennent pas de modifications (règles 70.16 et 70.17.)*) :

**Description, pages:**

1-52                      version initiale

**Revendications, N°:**

1-33                      version initiale

**Dessins, feuilles:**

1/3-3/3                      version initiale

2. En ce qui concerne la **langue**, tous les éléments indiqués ci-dessus étaient à la disposition de l'administration ou lui ont été remis dans la langue dans laquelle la demande internationale a été déposée, sauf indication contraire donnée sous ce point.

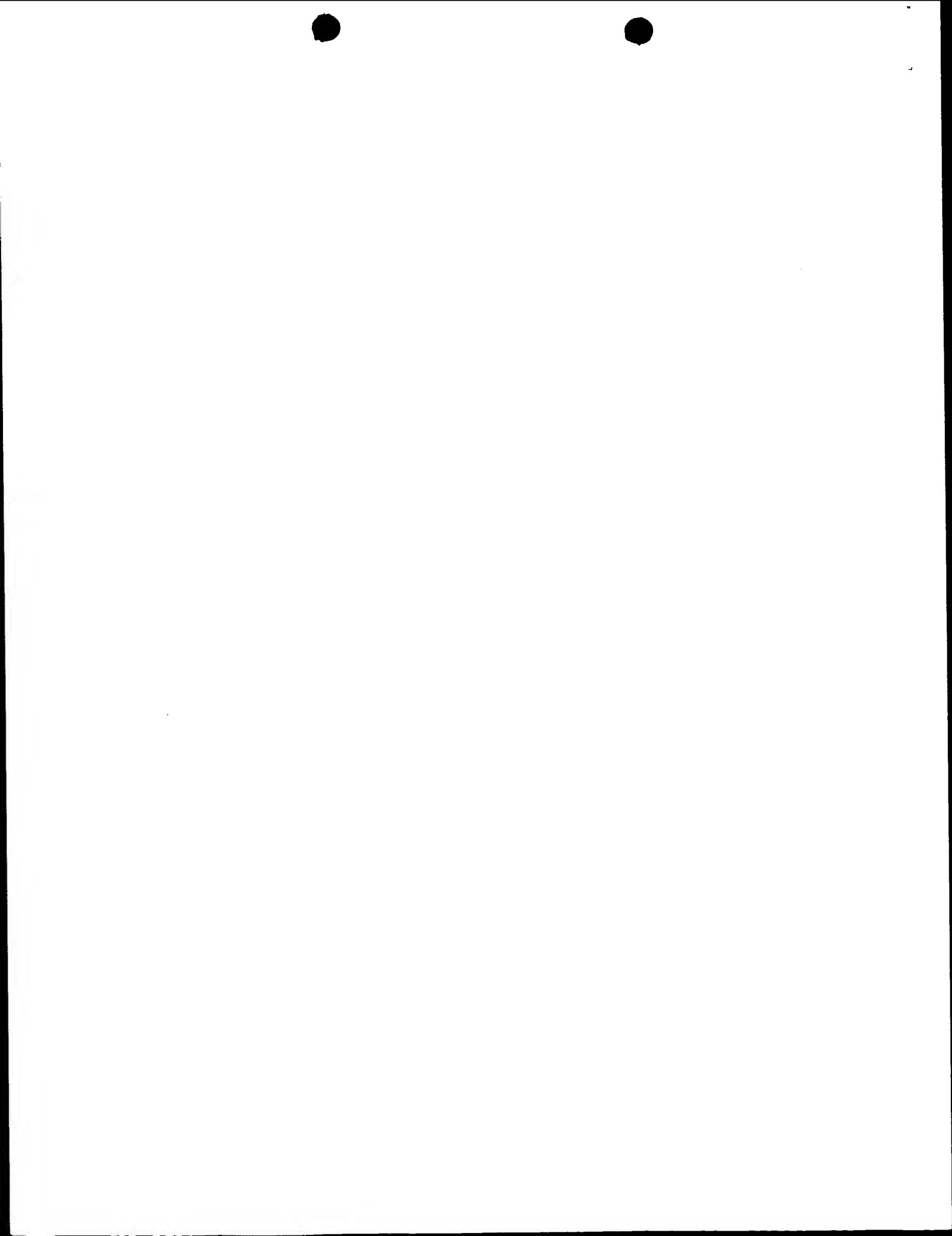
Ces éléments étaient à la disposition de l'administration ou lui ont été remis dans la langue suivante: , qui est :

- ☐ la langue d'une traduction remise aux fins de la recherche internationale (selon la règle 23.1(b)).
- ☐ la langue de publication de la demande internationale (selon la règle 48.3(b)).
- ☐ la langue de la traduction remise aux fins de l'examen préliminaire internationale (selon la règle 55.2 ou 55.3).

3. En ce qui concerne les **séquences de nucléotides ou d'acide aminés** divulguées dans la demande internationale (le cas échéant), l'examen préliminaire internationale a été effectué sur la base du listage des séquences :

- ☐ contenu dans la demande internationale, sous forme écrite.
- ☐ déposé avec la demande internationale, sous forme déchiffrable par ordinateur.
- ☐ remis ultérieurement à l'administration, sous forme écrite.
- ☐ remis ultérieurement à l'administration, sous forme déchiffrable par ordinateur.
- ☐ La déclaration, selon laquelle le listage des séquences par écrit et fourni ultérieurement ne va pas au-delà de la divulgation faite dans la demande telle que déposée, a été fournie.
- ☐ La déclaration, selon laquelle les informations enregistrées sous déchiffrable par ordinateur sont identiques à celles du listage des séquences Présenté par écrit, a été fournie.

4. Les modifications ont entraîné l'annulation :



**RAPPORT D'EXAMEN  
PRÉLIMINAIRE INTERNATIONAL**

Demande internationale n° PCT/FR99/02995

- ☐ de la description, pages :
- ☐ des revendications, n°s :
- ☐ des dessins, feuilles :

5. ☐ Le présent rapport a été formulé abstraction faite (de certaines) des modifications, qui ont été considérées comme allant au-delà de l'exposé de l'invention tel qu'il a été déposé, comme il est indiqué ci-après (règle 70.2(c)) :

*(Toute feuille de remplacement comportant des modifications de cette nature doit être indiquée au point 1 et annexée au présent rapport)*

6. Observations complémentaires, le cas échéant :

**III. Absence de formulation d'opinion quant à la nouveauté, l'activité inventive et la possibilité d'application industrielle**

1. La question de savoir si l'objet de l'invention revendiquée semble être nouveau, impliquer une activité inventive (ne pas être évident) ou être susceptible d'application industrielle n'a pas été examinée pour ce qui concerne :

- ☐ l'ensemble de la demande internationale.
- ☒ les revendications n°s 31-33 (voir application industrielle).

parce que :

- ☒ la demande internationale, ou les revendications n°s 31-33 (voir application industrielle) en question, se rapportent à l'objet suivant, à l'égard duquel l'administration chargée de l'examen préliminaire international n'est pas tenue effectuer un examen préliminaire international (*préciser*) :  
**voir feuille séparée**
  - ☐ la description, les revendications ou les dessins (*en indiquer les éléments ci-dessous*), ou les revendications n°s en question ne sont pas clairs, de sorte qu'il n'est pas possible de formuler une opinion valable (*préciser*) :
  - ☐ les revendications, ou les revendications n°s en question, ne se fondent pas de façon adéquate sur la description, de sorte qu'il n'est pas possible de formuler une opinion valable.
  - ☐ il n'a pas été établi de rapport de recherche internationale pour les revendications n°s en question.
2. Le listage des séquences de nucléotides ou d'acides aminés n'est pas conforme à la norme prévue dans l'annexe C des instructions administratives, de sorte qu'il n'est pas possible d'effectuer un examen préliminaire international significatif:
- ☐ le listage présenté par écrit n'a pas été fourni ou n'est pas conforme à la norme.
  - ☐ le listage sous forme déchiffrable par ordinateur n'a pas été fourni ou n'est pas conforme à la norme.



**RAPPORT D'EXAMEN  
PRÉLIMINAIRE INTERNATIONAL**

Demande internationale n° PCT/FR99/02995

**V. Déclaration motivée selon l'article 35(2) quant à la nouveauté, l'activité inventive et la possibilité d'application industrielle; citations et explications à l'appui de cette déclaration**

**1. Déclaration**

Nouveauté	Oui : Revendications 1-33
	Non : Revendications
Activité inventive	Oui : Revendications 1-33
	Non : Revendications
Possibilité d'application industrielle	Oui : Revendications 1-30
	Non : Revendications

**2. Citations et explications  
voir feuille séparée**

**VII. Irrégularités dans la demande internationale**

Les irrégularités suivantes, concernant la forme ou le contenu de la demande internationale, ont été constatées :  
**voir feuille séparée**



**Concernant le point III**

**Absence de formulation d'opinion quant à la nouveauté, l'activité inventive et la possibilité d'application industrielle**

Il n'existe pas de critère unifié dans les Etats parties au PCT pour déterminer si les revendications 31-33 sont susceptibles d'application industrielle. La brevetabilité peut aussi dépendre de la manière dont les revendications ont été formulées. Ainsi, l'Office européen des brevets ne considère pas comme susceptible d'application industrielle l'objet de revendications d'utilisation d'un composé à des fins médicales. Par contre, peuvent être acceptées des revendications relatives à un composé connu, pour une première utilisation à des fins médicales ainsi que des revendications relatives à l'utilisation d'un tel composé dans la fabrication d'un médicament en vue d'un nouveau traitement médical.

**Concernant le point V**

**Déclaration motivée selon l'article 35(2) quant à la nouveauté, l'activité inventive et la possibilité d'application industrielle; citations et explications à l'appui de cette déclaration**

1. Il est fait référence aux documents suivants:

- D1: FUHRHOP J -H ET AL: 'BOLAAMPHIPHILES WITH MANNOSE- AND TETRAALKYLAMMONIUM HEAD GROUPS ASCOATINGS FOR NUCLEIC ACIDS AND POSSIBLE REAGENTS FOR TRANSFECTIONS' CHEMISTRY AND PHYSICS OF LIPIDS, vol. 43, 1 avril 1987 (1987-04-01), pages 193-213, ISSN: 0009-3084
- D2: WO 96 25508 A (RHONE POULENC RORER SA ;BYK GERARDO (FR); SCHERMAN DANIEL (FR); SC) 22 août 1996 (1996-08-22) **cité dans la demande**
- D3: WO 98 06869 A (PHILADELPHIA CHILDREN HOSPITAL) 19 février 1998 (1998-02-19)
- D4: WO 96 21036 A (VIAGENE INC) 11 juillet 1996 (1996-07-11)
- D5: DATABASE CHEMICAL ABSTRACTS, abstract no. 126:207866, ERBACHER ET AL: 'THE REDUCTION OF THE POSITIVE CHARGES OF





POLYLYSINE BY PARTIAL GLUCONOYLATION INCREASES THE TRANSFECTION EFFICIENCY OF POLYLYSINE/DNA COMPLEXES' & BIOCHIM. BIOPHYS. ACTA, vol. 1324, no. 1, 1997, pages 27-36,

D6: DATABASE MEDLINE US NATIONAL LIBRARY OF MEDICINE (NLM), BETHESDA, MD, US; abstract 1999129193, GOULA ET AL:

'POLYETHYLENIMINE-BASED INTRAVENOUS DELIVERY OF TRANSGENES TO MOUSE LUNG' & GENE THERAPY, vol. 5, no. 9, septembre 1998 (1998-09), pages 1291-1295,

D7: DATABASE MEDLINE US NATIONAL LIBRARY OF MEDICINE (NLM), BETHESDA, MD, US; ABSTRACT 96200295, OKU ET AL: 'EFFECT OF SERUM PROTEIN BINDING ON REAL-TIME TRAFFICKING OF LIPOSOMES WITH DIFFERENT CHARGES ANALYZED BY POSITRON EMISSION TOMOGRAPHY' & BIOCHIMICA ET BIOPHYSICA ACTA, vol. 1280, no. 1, 3 avril 1996 (1996-04-03), pages 149-154.

2. La présente demande se rapporte entre-autres à des agents de transferts d'acides nucléiques constitués d'un **polycation** lié covalamment à 2 chaînes grasses hydrocarbonnés dont l'une au moins est liée en son extrémité à un sucre.
3. D1 divulgue des agents de transferts, qui eux comprennent un espaceur hydrophobe lié chimiquement d'une part à un **monocation** et d'autre part à au moins un substituant hydrophile (voir p. ex. composés 1b, 1d et 2b, à la page 195).
4. D2 décrit des polycations, de préférences des peptides dérivés d'une histone, d'une nucléine ou d'une protamine, c'est-à-dire des peptides de nature basique dans des compositions contenant un agent de transfection et un acide nucléique. Ces polycations peuvent comprendre une région lipophile (voir p. ex. revendication 25), sans qu'un espaceur hydrophobe lié chimiquement d'une part à un polycation et d'autre part à au moins un substituant hydrophile ne soit proposé.
5. D3 décrit des agents de transfert à base de polylysine glycosilée, qui ne comprennent pas d'espaceur hydrophobe.
6. De même D4 décrit des agents de transfert à base d'un polycation lié à une glycol



sans contenir des espaceurs hydrophobes.

7. D5 décrit l'utilisation d'une polylysine substituée avec un résidu polyhydroxyalcanoyl comme agent de transfert d'acide nucléique, c'est-à-dire sans qu'un espaceur hydrophobe ne soit proposé.
8. D6 décrit la transfection *in vivo* d'une acide désoxyribonucléique par utilisation d'une imine de polyéthylène dans une solution aqueuse contenant 5% de glucose.
9. D7 discute l'effet de protéine du sérum sur des liposomes utilisées entre-autres comme agent de transfert d'acides nucléiques.
10. Il en résulte qu' aucun des documents D1 à D7 ni divulgue, ni suggère la combinaison spécifique d'un espaceur hydrophobe lié chimiquement d'une part à un **polycation** et d'autre part à au moins un substituant hydrophile est sont utilisation comme agent de transfert d'acides nucléiques.

#### Concernant le point VII

#### **Irrégularités dans la demande internationale**

Contrairement à ce qu'exige la règle 5.1 a) ii) PCT, la description n'indique pas l'état de la technique antérieure pertinent exposé dans le document D1 et ne cite pas ce document.



## RAPPORT DE RECHERCHE INTERNATIONALE

Dr de Internationale No

PCT/FR 96/00248

Document brevet cité au rapport de recherche	Date de publication	Membre(s) de la famille de brevet(s)	Date de publication
WO-A-9117773	28-11-91	DE-A- 4110410	01-10-92
		AT-T- 126442	15-09-95
		DE-D- 59106279	21-09-95
		EP-A- 0532525	24-03-93
		ES-T- 2078521	16-12-95
-----			
EP-A-388758	26-09-90	AU-B- 637085	20-05-93
		AU-B- 5137290	20-09-90
		CA-A- 2012311	16-09-90
		IL-A- 93755	31-12-95
		JP-A- 3200800	02-09-91
		US-A- 5354844	11-10-94
-----			
WO-A-9319768	14-10-93	AU-B- 4027893	08-11-93
		CA-A- 2133323	14-10-93
		EP-A- 0636028	01-02-95
		JP-T- 7505639	22-06-95
-----			

# RAPPORT DE RECHERCHE INTERNATIONALE

Demande internationale No. PCT/FR96/00248

## SUITE DES RENSEIGNEMENTS INDIQUÉS SUR PCT/ISA/210

Remarque: Bien que les revendications 36-38 concernent (pour autant in vivo) une méthode de traitement du corps humain/animal (Regle 39.1 (iv)), la recherche a été effectuée et basée sur les effets imputés au produit (à la composition).

# RAPPORT DE RECHERCHE INTERNATIONALE

Demande internationale n°

PCT/FR96/00248

## Cadre I Observations - lorsqu'il a été estimé que certaines revendications ne pouvaient pas faire l'objet d'une recherche (suite du point 1 de la première feuille)

Conformément à l'article 17.2)a), certaines revendications n'ont pas fait l'objet d'une recherche pour les motifs suivants:

1. ☒ Les revendications n<sup>os</sup> se rapportent à un objet à l'égard duquel l'administration n'est pas tenue de procéder à la recherche, à savoir:

Voir annexe.

2. ☐ Les revendications n<sup>os</sup> se rapportent à des parties de la demande internationale qui ne remplissent pas suffisamment les conditions prescrites pour qu'une recherche significative puisse être effectuée, en particulier:

3. ☐ Les revendications n<sup>os</sup> sont des revendications dépendantes et ne sont pas rédigées conformément aux dispositions de la deuxième et de la troisième phrases de la règle 6.4.a).

## Cadre II Observations - lorsqu'il y a absence d'unité de l'invention (suite du point 2 de la première feuille)

L'administration chargée de la recherche internationale a trouvé plusieurs inventions dans la demande internationale, à savoir:

1. ☐ Comme toutes les taxes additionnelles ont été payées dans les délais par le déposant, le présent rapport de recherche internationale porte sur toutes les revendications pouvant faire l'objet d'une recherche.
2. ☐ Comme toutes les recherches portant sur les revendications qui s'y prêtaient ont pu être effectuées sans effort particulier justifiant une taxe additionnelle, l'administration n'a sollicité le paiement d'aucune taxe de cette nature.
3. ☐ Comme une partie seulement des taxes additionnelles demandées a été payée dans les délais par le déposant, le présent rapport de recherche internationale ne porte que sur les revendications pour lesquelles les taxes ont été payées, à savoir les revendications n<sup>os</sup>:
4. ☐ Aucune taxe additionnelle demandée n'a été payée dans les délais par le déposant. En conséquence, le présent rapport de recherche internationale ne porte que sur l'invention mentionnée en premier lieu dans les revendications; elle est couverte par les revendications n<sup>os</sup>:

Remarque quant à la réserve

- ☐ Les taxes additionnelles étaient accompagnées d'une réserve de la part du déposant.
- ☐ Le paiement des taxes additionnelles n'était assorti d'aucune réserve.

# RAPPORT DE RECHERCHE INTERNATIONALE

Document Internationale No

PCT/FR 96/00248

C.(suite) DOCUMENTS CONSIDERES COMME PERTINENTS		
Catégorie *	Identification des documents cités, avec, le cas échéant, l'indication des passages pertinents	no. des revendications visées
X	WO,A,91 17773 (BOEHRINGER INGELHEIM INTERNATIONAL GNBH & GENENTECH INC.) 28 Novembre 1991 voir revendications 11-13 ---	1
X	EP,A,0 388 758 (THE BOEHRINGER INGELHEIM INTERNATIONAL GMBH) 26 Septembre 1990 voir revendications ---	1,13
A	JOURNAL OF VIROLOGICAL METHODS, vol. 23, no. 2, Février 1989, pages 187-194, XP002008073 CORNETTA, K. & ANDERSON, W.F.: "Protamine sulfate as an effective alternative to polybrene in retroviral-mediated gene transfer: implications for human gene therapy" voir le document en entier ---	1
X	PROTOPLASMA, vol. 96, 1978, pages 209-223, XP002008074 DUBES, G.R. & WEGRZYN, R.J.: "Rapid ephemeral cell sensitization as the mechanism of histone-induced and protamine-induced enhancement of transfection by poliovirus RNA" voir le document en entier ---	1
A	WO,A,93 19768 (THE REGENTS OF THE UNIVERSITY OF CALIFORNIA) 14 Octobre 1993 voir revendications -----	1



# RAPPORT DE RECHERCHE INTERNATIONALE

De: de Internationale No  
PCT/FR 96/00248

A. CLASSEMENT DE L'OBJET DE LA DEMANDE  
CIB 6 C12N15/87 A61K48/00

Selon la classification internationale des brevets (CIB) ou à la fois selon la classification nationale et la CIB

B. DOMAINES SUR LESQUELS LA RECHERCHE A PORTE

Documentation minimale consultée (système de classification suivi des symboles de classement)

CIB 6 C12N A61K

Documentation consultée autre que la documentation minimale dans la mesure où ces documents relèvent des domaines sur lesquels a porté la recherche

Base de données électronique consultée au cours de la recherche internationale (nom de la base de données, et si cela est réalisable, termes de recherche utilisés)

## C. DOCUMENTS CONSIDERES COMME PERTINENTS

Catégorie *	Identification des documents cités, avec, le cas échéant, l'indication des passages pertinents	no. des revendications visées
X	BIOCHIMICA AND BIOPHYSICA ACTA, vol. 950, no. 2, 13 Juillet 1988, pages 221-228, XP002008071 BOETTGER, M. ET AL.: "Condensation of vector DNA by the chromosomal protein HMG1 results in efficient transfection" voir le document en entier ---	1
X	DNA, vol. 6, no. 1, Février 1987, pages 81-89, XP002008072 WIERNHUES, U. ET AL.: "A novel method for transfection and expression of reconstituted DNA-protein complexes in eukaryotic cells" voir le document en entier --- -/--	1

☒ Voir la suite du cadre C pour la fin de la liste des documents

☒ Les documents de familles de brevets sont indiqués en annexe

\* Catégories spéciales de documents cités:

- "A" document définissant l'état général de la technique, non considéré comme particulièrement pertinent
- "E" document antérieur, mais publié à la date de dépôt international ou après cette date
- "L" document pouvant jeter un doute sur une revendication de priorité ou cité pour déterminer la date de publication d'une autre citation ou pour une raison spéciale (telle qu'indiquée)
- "O" document se référant à une divulgation orale, à un usage, à une exposition ou tous autres moyens
- "P" document publié avant la date de dépôt international, mais postérieurement à la date de priorité revendiquée

- "T" document ultérieur publié après la date de dépôt international ou la date de priorité et n'appartenant pas à l'état de la technique pertinent, mais cité pour comprendre le principe ou la théorie constituant la base de l'invention
- "X" document particulièrement pertinent; l'invention revendiquée ne peut être considérée comme nouvelle ou comme impliquant une activité inventive par rapport au document considéré isolément
- "Y" document particulièrement pertinent; l'invention revendiquée ne peut être considérée comme impliquant une activité inventive lorsque le document est associé à un ou plusieurs autres documents de même nature, cette combinaison étant évidente pour une personne du métier
- "&" document qui fait partie de la même famille de brevets

Date à laquelle la recherche internationale a été effectivement achevée

11 Juillet 1996

Date d'expédition du présent rapport de recherche internationale

23.07.96

Nom et adresse postale de l'administration chargée de la recherche internationale  
Office Européen des Brevets, P.B. 5818 Patentlaan 2  
NL - 2280 HV Rijswijk  
Tel. (+ 31-70) 340-2040, Tx. 31 651 epo nl,  
Fax: (+ 31-70) 340-3016

Fonctionnaire autorisé

Chambonnet, F

# INTERNATIONAL SEARCH REPORT

International Application No  
PCT/FR 96/00248

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO-A-9117773	28-11-91	DE-A- 4110410	01-10-92
		AT-T- 126442	15-09-95
		DE-D- 59106279	21-09-95
		EP-A- 0532525	24-03-93
		ES-T- 2078521	16-12-95
-----			
EP-A-388758	26-09-90	AU-B- 637085	20-05-93
		AU-B- 5137290	20-09-90
		CA-A- 2012311	16-09-90
		IL-A- 93755	31-12-95
		JP-A- 3200800	02-09-91
		US-A- 5354844	11-10-94
-----			
WO-A-9319768	14-10-93	AU-B- 4027893	08-11-93
		CA-A- 2133323	14-10-93
		EP-A- 0636028	01-02-95
		JP-T- 7505639	22-06-95
-----			

# INTERNATIONAL SEARCH REPORT

International application No.  
PCT/FR96/00248

**Comment:** although claims 36-38 concern an (in vivo) method for treatment of the human or animal body (PCT Rule 39.1(iv)), the search has been based on the effects attributed to the products (composition).

# INTERNATIONAL SEARCH REPORT

International application No.  
PCT/FR96/00248

## Box I Observations where certain claims were found unsearchable (Continuation of Item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.:  
because they relate to subject matter not required to be searched by this Authority, namely:  
See annex.
2. ☐ Claims Nos.:  
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

## Box II Observations where unity of invention is lacking (Continuation of Item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

### Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.  
☐ No protest accompanied the payment of additional search fees.

# INTERNATIONAL SEARCH REPORT

International Application No.  
PCT/FR 96/00248

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO,A,91 17773 (BOEHRINGER INGELHEIM INTERNATIONAL GNBH & GENENTECH INC.) 28 November 1991 see claims 11-13 ---	1
X	EP,A,0 388 758 (THE BOEHRINGER INGELHEIM INTERNATIONAL GMBH) 26 September 1990 see claims ---	1,13
A	JOURNAL OF VIROLOGICAL METHODS, vol. 23, no. 2, February 1989, pages 187-194, XP002008073 CORNETTA, K. & ANDERSON, W.F.: "Protamine sulfate as an effective alternative to polybrene in retroviral-mediated gene transfer: implications for human gene therapy" see the whole document ---	1
X	PROTOPLASMA, vol. 96, 1978, pages 209-223, XP002008074 DUBES, G.R. & WEGRZYN, R.J.: "Rapid ephemeral cell sensitization as the mechanism of histone-induced and protamine-induced enhancement of transfection by poliovirus RNA" see the whole document ---	1
A	WO,A,93 19768 (THE REGENTS OF THE UNIVERSITY OF CALIFORNIA) 14 October 1993 see claims -----	1

# INTERNATIONAL SEARCH REPORT

International Application No  
PCT/FR 96/00248

A. CLASSIFICATION OF SUBJECT MATTER  
IPC 6 C12N15/87 A61K48/00

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)  
IPC 6 C12N A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	BIOCHIMICA AND BIOPHYSICA ACTA, vol. 950, no. 2, 13 July 1988, pages 221-228, XP002008071 BOETTGER, M. ET AL.: "Condensation of vector DNA by the chromosomal protein HMG1 results in efficient transfection" see the whole document ---	1
X	DNA, vol. 6, no. 1, February 1987, pages 81-89, XP002008072 WIERNHUES, U. ET AL.: "A novel method for transfection and expression of reconstituted DNA-protein complexes in eukaryotic cells" see the whole document --- -/-	1

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

### \* Special categories of cited documents:

- \*A\* document defining the general state of the art which is not considered to be of particular relevance
- \*E\* earlier document but published on or after the international filing date
- \*L\* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- \*O\* document referring to an oral disclosure, use, exhibition or other means
- \*P\* document published prior to the international filing date but later than the priority date claimed

- \*T\* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- \*X\* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- \*Y\* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
- \*&\* document member of the same patent family

Date of the actual completion of the international search

11 July 1996

Date of mailing of the international search report

23.07.96

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2  
NL - 2280 HV Rijswijk  
Tel. (+ 31-70) 340-2040, Tx. 31 651 epo nl,  
Fax (+ 31-70) 340-3016

Authorized officer

Chambonnet, F

37. Utilisation d'un composé constitué en tout ou partie, de motifs peptidiques (KTPKKAKKP) et/ou (ATPAKKAA) avec le nombre de ces motifs pouvant varier entre 2 et 10, pour, lorsqu'il est couplé à un ligand de récepteur cellulaire, un anticorps ou dérivé d'anticorps, cibler, in vitro, ex vivo et/ou in vivo, un acide nucléique vers des
- 5 cellules exprimant les récepteurs ou anti-gènes correspondants.

38. Utilisation d'un oligopeptide sélectionné parmi :
- (ATPAKKAA)<sub>2</sub>(COOH),  
(KTPKKAKKP)<sub>2</sub>(COOH),  
ATPKKSAKKTPKKAKKP(COOH),  
10 KKAKSPKKAKAAKPKKAPKSPAKAKA(COOH),  
SRSRYRQRQSRRRRRR(COOH). et  
RRRLHRIHRRQHRSCRRRKRR(COOH).  
pour effectuer le transfert in vitro, ex vivo et/ou in vivo d'au moins un acide nucléique,  
ledit oligonucléotide étant associé ou non à un élément de ciblage.

27. Composition pharmaceutique selon l'une quelconque des revendications 1 à 18 et 23 à 26 caractérisée en ce que l'agent de transfection est la dioctadécylamidoglycyl spermine (DOGS).

5 28. Composition pharmaceutique selon l'une des revendications précédentes caractérisée en ce que l'acide nucléique est un acide désoxyribonucléique.

29. Composition pharmaceutique selon l'une des revendications 1 à 27 caractérisée en ce que l'acide nucléique est un acide ribonucléique.

30. Composition pharmaceutique selon la revendication 28 ou 29 caractérisée en ce que l'acide nucléique est modifié chimiquement.

10 31. Composition pharmaceutique selon la revendication 28, 29 ou 30 caractérisée en ce que l'acide nucléique est un antisens.

32. Composition pharmaceutique selon l'une des revendications 28 à 30 caractérisée en ce que l'acide nucléique comporte un gène thérapeutique.

15 33. Composition pharmaceutique selon l'une des revendications précédentes comprenant en outre un ou plusieurs lipides neutres.

34. Composition pharmaceutique selon la revendication 33 caractérisée en ce que le ou les lipides neutres sont choisis parmi les lipides synthétiques ou naturels, zwitterioniques ou dépourvus de charge ionique dans les conditions physiologiques.

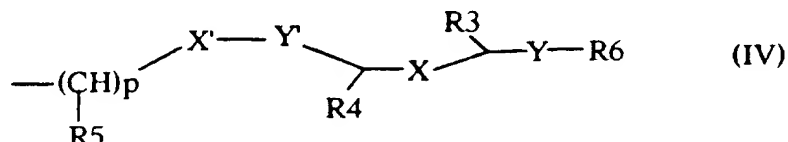
20 35. Composition pharmaceutique selon la revendication 33 ou 34 caractérisée en ce que le ou les lipides neutres sont choisis parmi la dioléoylphosphatidyléthanamine (DOPE), l'oléoyl-palmitoylphosphatidyléthanamine (POPE), le distéaroyl, -palmitoyl, -mirystoyl phosphatidyléthanamine ainsi que leurs dérivé N-méthylés 1 à 3 fois; les phosphatidylglycérols, les diacylglycérols, les glycosyldiacylglycérols, les cérebrosides (tels que notamment les galactocérebrosides),  
25 les sphingolipides (tels que notamment les sphingomyélines) et les asialogangliosides (tels que notamment les asialoGM1 et GM2).

36. Utilisation d'une composition pharmaceutique selon l'une des revendications 1 à 35 pour le transfert in vitro, ex vivo et/ou in vivo d'acides nucléiques.



24. Composition pharmaceutique selon la revendication 23 ou 24 caractérisée en ce que la région polyamine est représentée par la spermine, la thermine ou un de leurs analogues ayant conservé ses propriétés de liaison à l'acide nucléique.

25. Composition pharmaceutique selon la revendication 23 caractérisée en ce que le lipofectant comprend au moins une région lipophile représentée par la formule générale (IV)



dans laquelle

- X et X' représentent, indépendamment l'un de l'autre, un atome d'oxygène, un groupement méthylène  $-(\text{CH}_2)_q-$  avec q égal à 0, 1, 2 ou 3, ou un groupement amino

$-\text{NH}-$  ou  $-\text{NR}'-$  avec R' représentant un groupement alkyle en C<sub>1</sub> à C<sub>4</sub>,

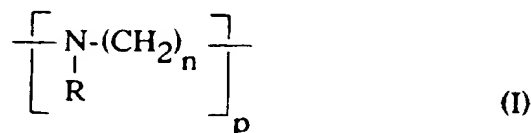
- Y et Y' représentent indépendamment l'un de l'autre un groupement méthylène, un groupement carbonyle ou un groupement C=S,

- R<sub>3</sub>, R<sub>4</sub> et R<sub>5</sub> représentent indépendamment l'un de l'autre un atome d'hydrogène ou un radical alkyle, substitué ou non, en C<sub>1</sub> à C<sub>4</sub>, avec p pouvant varier entre 0 et 5,

- R<sub>6</sub> représente un dérivé du cholestérol ou un groupement alkyle amino - NR<sub>1</sub>R<sub>2</sub> avec R<sub>1</sub> et R<sub>2</sub> représentant indépendamment l'un de l'autre un radical aliphatique, saturé ou non, linéaire ou ramifié en C<sub>12</sub> à C<sub>22</sub>.

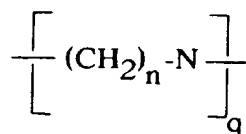
26. Composition pharmaceutique selon la revendication 23 caractérisée en ce qu'il s'agit de préférence d'une lipopolyamine choisie parmi la dioctadécylamidoglycyl spermine (DOGS) la 5-carboxyspermylamide de la palmitoylphosphatidylethanolamine (DPPES), le (Dioctadécyl-carbamoylméthoxy)-acétate de 2-5-bis-(3-amino-propylamino)-pentyle ou le (Dioctadécyl-carbamoylméthoxy)-acétate de 1,3-bis-(3-amino-propylamino)-2 propyle, le  $\{\text{H}_2\text{N}(\text{CH}_2)_3\}_2\text{N}(\text{CH}_2)_4\text{N}\{(\text{CH}_2)_3\text{NH}_2\}(\text{CH}_2)_3\text{NHCH}_2\text{COGlyN}[(\text{CH}_2)_{17}\text{-CH}_3]_2$ , le  $\text{H}_2\text{N}(\text{CH}_2)_3\text{NH}(\text{CH}_2)_4\text{NH}(\text{CH}_2)_3\text{NHCH}_2\text{COGlyN}[(\text{CH}_2)_{18}]_2$  ou le  $\text{H}_2\text{N}(\text{CH}_2)_3\text{NH}(\text{CH}_2)_4\text{NH}(\text{CH}_2)_3\text{NHCH}_2\text{COArgN}[(\text{CH}_2)_{18}]$ .

20. Composition pharmaceutique selon la revendication 18 caractérisée en ce que le polymère cationique est de préférence un composé de formule générale (I) :



dans laquelle

- 5 - R peut être un atome d'hydrogène ou un groupe de formule



- n est un nombre entier compris entre 2 et 10;

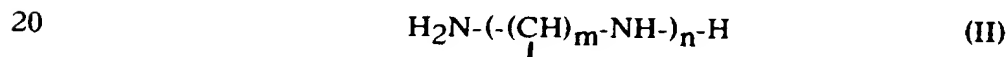
- p et q sont des nombres entiers,

- 10 avec la somme p+q étant telle que le poids moléculaire moyen du polymère soit compris entre 100 et 10<sup>7</sup>.

21. Composition pharmaceutique selon la revendication 18 caractérisée en ce que le polymère cationique est choisi parmi le polyéthylène imine (PEI) et le polypropylène imine (PPI).

- 15 22. Composition pharmaceutique selon la revendication 21 caractérisée en ce que le polymère est choisi parmi le polyéthylène imine de poids moléculaire moyen 50 000 (PEI50K) et le polyéthylène imine de poids moléculaire moyen 800 000 (PEI800K).

23. Composition pharmaceutique selon la revendication 18 caractérisée en ce que le lipofectant comprend au moins une région polyamine de formule générale (II)



- 25 dans laquelle m est un nombre entier supérieur ou égal à 2 et n est un nombre entier supérieur ou égal à 1, m pouvant varier entre les différents groupes de carbone compris entre 2 amines associée de manière covalente à une région lipophile de type chaîne hydrocarbonée, saturée ou non, du cholestérol, ou un lipide naturel ou synthétique capable de former des phases lamellaires ou hexagonales.

9. Composition pharmaceutique selon la revendication 8 caractérisée en ce qu'il s'agit du peptide (ATPAKKAA)<sub>2</sub>(COOH).

10. Composition pharmaceutique selon la revendication 1 caractérisé en ce que le composé dérive de la protamine.

5 11. Composition pharmaceutique selon la revendication 10 caractérisé en ce qu'il s'agit d'un oligopeptide choisi parmi SRSRYRQRQSRRRRRR(COOH) et RRRLHRIHRRQHRSCRRRKRR(COOH).

12. Composition pharmaceutique selon l'une des revendications précédentes caractérisée en ce que ledit composé possède une structure en feuillet  $\beta$ .

10 13. Composition pharmaceutique selon l'une des revendications 1 à 12 caractérisée en ce que ledit composé est en outre associé à un ligand de récepteur cellulaire.

14. Composition pharmaceutique selon la revendication 13 caractérisée en ce qu'il s'agit de tout ou partie de l'histone H1 associé à une séquence signal de  
15 localisation nucléaire.

15. Composition pharmaceutique selon la revendication 14 caractérisée en ce qu'il s'agit du peptide PKKKRKV-bAla-(KTPKKAKKP)<sub>2</sub>(COOH).

16. Composition pharmaceutique selon l'une des revendications 1 à 15 caractérisée en ce que ledit composé est en outre associé à un peptide de type  
20 fusogène favorisant la transfection cellulaire de ladite composition.

17. Composition pharmaceutique selon l'une des revendications 1 à 16 caractérisée en ce que ledit composé est en outre polyglycosylé, sulfoné, phosphorylé et/ou greffé à des sucres complexes ou à un agent lipophile.

18. Composition pharmaceutique selon l'une des revendications précédentes  
25 caractérisée en ce que l'agent de transfection est un polymère cationique ou un lipofectant.

19. Composition pharmaceutique selon la revendication 18 caractérisée en ce que le lipofectant est un lipide susceptible de former des lipisomes, des liposomes furtifs, des immunoliposomes ou des liposomes ciblés.

### REVENDICATIONS

1. Composition pharmaceutique utile pour la transfection d'un acide nucléique caractérisée en ce qu'elle contient outre ledit acide nucléique, au moins un agent de transfection et un composé intervenant au niveau de la condensation dudit acide nucléique, ledit composé dérivant en tout ou partie d'une histone, d'une nucléoline d'une protamine et/ou de l'un de leurs dérivés.  
5
2. Composition pharmaceutique utile pour la transfection d'un acide nucléique contenant outre ledit acide nucléique, un agent de transfection et au moins un composé intervenant au niveau de la condensation dudit acide nucléique caractérisée en ce que ledit composé est constitué en tout ou partie, de motifs peptidiques (KTPKKAKKP) et/ou (ATPAKKAA) répétés de manière continue ou non, le nombre des motifs pouvant varier entre 2 et 10.  
10
3. Composition pharmaceutique selon la revendication 2 caractérisée en ce que les motifs peptidiques peuvent être séparés entre eux par des liens de nature biochimique de type acides aminés et/ou de nature chimique.  
15
4. Composition pharmaceutique selon la revendication 3 caractérisée en ce qu'il s'agit de liens constitués d'un ou plusieurs acides aminés.
5. Composition pharmaceutique selon la revendication 1 à 4 caractérisée en ce que le composé dérive de l'histone H1.
- 20 6. Composition pharmaceutique selon la revendication 5 caractérisée en ce que le composé dérive du domaine C- terminal de l'histone H1.
7. Composition pharmaceutique selon la revendication 5 ou 6 caractérisée en ce qu'il s'agit de préférence d'un oligopeptide choisi parmi (KTPKKAKKP)<sub>2</sub>(COOH),  
25 ATPKKSAKKTPKKAKKP(COOH), et KKA<sub>2</sub>SPKKA<sub>2</sub>AAKPKKAPKSPAKAKA(COOH).
8. Composition pharmaceutique selon l'une des revendications 1 à 4 caractérisée en ce que le composé dérive du domaine N- terminal de la nucléoline.

Arg Arg Arg Leu His Arg Ile His Arg Arg Gln His Arg Ser Cys Arg Arg  
5 10 15

Arg Lys Arg Arg  
5 20

10 (2) INFORMATIONS POUR LA SEQ ID NO: 9:

(i) CARACTERISTIQUES DE LA SEQUENCE:  
(A) LONGUEUR: 26 acides aminés  
(B) TYPE: acide aminé  
(D) CONFIGURATION: linéaire

(ii) TYPE DE MOLECULE: protéine

(xi) DESCRIPTION DE LA SEQUENCE: SEQ ID NO: 9:

Pro Lys Lys Lys Arg Lys Val Ala Lys Thr Pro Lys Lys Ala Lys Lys Pro  
5 10 15

Lys Thr Pro Lys Lys Ala Lys Lys Pro  
 20 25  
 25

## 5 (2) INFORMATIONS POUR LA SEQ ID NO: 6:

## (i) CARACTERISTIQUES DE LA SEQUENCE:

(A) LONGUEUR: 26 acides aminés

10 (B) TYPE: acide aminé

(D) CONFIGURATION: linéaire

(ii) TYPE DE MOLECULE: protéine

## 15 (xi) DESCRIPTION DE LA SEQUENCE: SEQ ID NO: 6:

Lys Lys Ala Lys Ser Pro Lys Lys Ala Lys Ala Ala Lys Pro Lys Lys Ala

5

10

15

Pro Lys Ser Pro Ala Lys Ala Lys Ala

20

25

20

## 25 (2) INFORMATIONS POUR LA SEQ ID NO: 7:

## (i) CARACTERISTIQUES DE LA SEQUENCE:

(A) LONGUEUR: 18 acides aminés

(B) TYPE: acide aminé

30 (D) CONFIGURATION: linéaire

(ii) TYPE DE MOLECULE: protéine

## 35 (xi) DESCRIPTION DE LA SEQUENCE: SEQ ID NO: 7:

Ser Arg Ser Arg Tyr Tyr Arg Gln Arg Gln Arg Ser Arg Arg Arg Arg Arg

5

10

15

Arg

40

## (2) INFORMATIONS POUR LA SEQ ID NO: 8:

## 45 (i) CARACTERISTIQUES DE LA SEQUENCE:

(A) LONGUEUR: 21 acides aminés

(B) TYPE: acide aminé

(D) CONFIGURATION: linéaire

50 (ii) TYPE DE MOLECULE: protéine

## (xi) DESCRIPTION DE LA SEQUENCE: SEQ ID NO: 8:

- 5 (2) INFORMATIONS POUR LA SEQ ID NO: 3:
- 10 (i) CARACTERISTIQUES DE LA SEQUENCE:  
(A) LONGUEUR: 16 acides aminés  
(B) TYPE: acide aminé  
(D) CONFIGURATION: linéaire
- 15 (ii) TYPE DE MOLECULE: protéine
- (xi) DESCRIPTION DE LA SEQUENCE: SEQ ID NO: 3:
- Ala Thr Pro Ala Lys Lys Ala Ala Ala Thr Pro Ala Lys Lys Ala Ala  
5 10 15
- 20 (2) INFORMATIONS POUR LA SEQ ID NO: 4:
- 25 (i) CARACTERISTIQUES DE LA SEQUENCE:  
(A) LONGUEUR: 18 acides aminés  
(B) TYPE: acide aminé  
(D) CONFIGURATION: linéaire
- (ii) TYPE DE MOLECULE: protéine
- 30 (xi) DESCRIPTION DE LA SEQUENCE: SEQ ID NO: 4:
- Lys Thr Pro Lys Lys Ala Lys Lys Pro Lys Thr Pro Lys Lys Ala Lys  
5 10 15
- Lys Pro
- 35 (2) INFORMATIONS POUR LA SEQ ID NO: 5:
- 40 (i) CARACTERISTIQUES DE LA SEQUENCE:  
(A) LONGUEUR: 17 acides aminés  
(B) TYPE: acide aminé  
(D) CONFIGURATION: linéaire
- 45 (ii) TYPE DE MOLECULE: protéine
- (xi) DESCRIPTION DE LA SEQUENCE: SEQ ID NO: 5:
- 50 Ala Thr Pro Lys Lys Ser Ala Lys Lys Thr Pro Lys Lys Ala Lys Lys Pro  
5 10 15

## LISTE DE SEQUENCES

## 5 (1) INFORMATIONS GENERALES:

## (i) DEPOSANT:

- 10 (A) NOM: RHONE POULENC RORER S.A.  
(B) RUE: 20, Avenue Raymond Aron  
(C) VILLE: ANTONY  
(E) PAYS: FRANCE  
(F) CODE POSTAL: 92165  
(G) TELEPHONE: 40.91.69.22  
15 (H) TELECOPIE: (1) 40.91.72.96

(ii) TITRE DE L' INVENTION: COMPOSITION CONTENANT DES ACIDES  
NUCLEIQUES, PREPARATION ET UTILISATION

20 (iii) NOMBRE DE SEQUENCES: 9

(iv) FORME DECHIFFRABLE PAR ORDINATEUR:

- (A) TYPE DE SUPPORT: Tape  
(B) ORDINATEUR: IBM PC compatible  
(C) SYSTEME D' EXPLOITATION: PC-DOS/MS-DOS  
25 (D) LOGICIEL: PatentIn Release #1.0, Version #1.30 (OEB)

## (2) INFORMATIONS POUR LA SEQ ID NO: 1:

## (i) CARACTERISTIQUES DE LA SEQUENCE:

- 30 (A) LONGUEUR: 9 acides aminés  
(B) TYPE: acide aminé  
(D) CONFIGURATION: linéaire

35 (ii) TYPE DE MOLECULE: protéine

(xi) DESCRIPTION DE LA SEQUENCE: SEQ ID NO: 1:

Lys Thr Pro Lys Lys Ala Lys Lys Pro

5

## (2) INFORMATIONS POUR LA SEQ ID NO: 2:

## (i) CARACTERISTIQUES DE LA SEQUENCE:

- 45 (A) LONGUEUR: 8 acides aminés  
(B) TYPE: acide aminé  
(D) CONFIGURATION: linéaire

50 (ii) TYPE DE MOLECULE: protéine

(xi) DESCRIPTION DE LA SEQUENCE: SEQ ID NO: 2:

Ala Thr Pro Ala Lys Lys Ala Ala

55 5



Tampon	Peptide		Résultat, RLU /tumeur		Nbre d'animaux traités
	référence	pept/ADN w/w	moyenne	écart-type	
NaCl/gluc/ MES ou HEPES 5mM					
150/HEPES	nls-H	0,02	1 689 938 6 819 100	1 388 072 5 860 709	6 6
150/5/HEPES	nls-H1	0,1	369 563 1 654 313	577 901 2 145 147	6* 6*
	nls-H1	0,02	4 031 000	1 007 896	6*
	H	0,1	931 275 3 420 432	214 229 1 285 704	4 6

TABLEAU 13

\*: souris endormies pendant l'injection, par narco-neuroleptanalgie avec un mélange Imalgène+ Rompun (Ketamine 130 mg/kg, Xylasine 4 mg/kg, voie intra-péritonéale).

- 5 Ces résultats montrent que, dans des tumeurs susceptibles de pouvoir être transfectées par de l'ADN nu, l'addition de peptide avec des faibles rapport peptide/ADN, sans association de lipides cationiques, permet d'augmenter l'expression du gène exogène par rapport à l'ADN nu seul.

EXEMPLE 8:Injections in vivo de peptides compactants selon l'invention sans lipofectant dans des tumeurs

Modèle:

- 5 - souris de type Swiss/nude femelles adulte  
- tumeurs expérimentales induites après injection de  $10^7$  cellules 3T3 HER2 par voie sous-cutanée au niveau du flanc.  
- injection du mélange de transfection 7 à 12 jours après l'injection des cellules. La solution d'ADN compacté ou non à du peptide est injectée directement dans la tumeur  
10 avec une seringue de type Hamilton.  
- deux jours après l'injection, prélèvement du tissu tumoral, qui est pesé puis haché et homogénéisé dans 750  $\mu$ l tampon de lyse (Promega Cell Lysis Buffer E153 A). Après centrifugation (20 000 g pendant 10 minutes), on prélève 10  $\mu$ l qui servent à l'évaluation de l'activité luciférase par mesure de l'émission lumineuse totale obtenue  
15 après mélange avec 50  $\mu$ l de réactif (Promega Luciferase Assay Substrate ) dans un luminomètre Lumat LB 9501 (Berthold), intégration sur 10 secondes.

L'activité résultante est exprimée en RLU (Relative Lights Units) estimés dans la totalité du surnageant de lyse tumorale.

- Protocole: L'ADN est dilué à 0,5 mg/ml dans une solution qui contiendra en final les  
20 sels, le tampon et le glucose en quantité finale telle que mentionnée dans le tableau de résultats. Dans certains groupes, avant injection, du peptide en solution à 1 mg/ml dans l'eau est ajouté à l'ADN en quantité suffisante pour atteindre les rapports poids/poids indiqués. Une incubation d'au moins 20 minutes à température ambiante est réalisée. Les souris reçoivent une injection de 20  $\mu$ l, soit 10  $\mu$ g d'ADN au total par  
25 tumeur.

**EXEMPLE 7****Transfert d'acide nucléique in vitro dans des cellules 3LL**

Cet exemple décrit le transfert d'acides nucléiques in vitro (sur cultures cellulaires) au moyen d'une composition selon l'invention comprenant l'acide nucléique, un composé selon l'invention choisi parmi les dérivés de protamines et une lipopolyamine dans une solution de NaCl 75 mM final.

On prépare un mélange de 10 µl composé comme suit:

- 0.5 µg d'ADN plasmidique pCMV-luc, 0,5 µg de PR1 ou PR2 un composé selon l'invention, dans une solution de NaCl 75 mM final
- Lipopolyamine C (RPR 120535) dans des rapport de charges tels qu'indiqués dans chacun des essais répertoriés dans le tableau 11 ci-après.

1.10<sup>5</sup> cellules 3LL (dans 250 µl de milieu de culture DMEM avec 10 % de sérum de veau foetal) sont incubées avec le mélange précédent à 37°C sous une atmosphère de CO<sub>2</sub> à 5 % pendant 4 heures. 500 µl de milieu de culture sont ajoutés et les cellules sont remises en culture. Le lendemain, les cellules sont lavées et remises en culture pendant 24 heures dans le même milieu contenant 10 % de sérum de veau foetal.

Le tapis cellulaire est ensuite lysé dans 100 µl de tampon de lyse (Promega). L'activité luciférase est mesurée en ajoutant 50 µl de substrat (Promega). La lecture se fait sur le luminomètre LB en cumulant les RLU (relative Light Unit) sur 10 secondes.

Les tableaux 11 et 12 rendent compte respectivement des essais avec PR1 et PR2.

RAPPORT DE CHARGE Lipopolyamine C/ADN	SANS COMPOSE (RLU)	AVEC PR2 (RLU)
4X	107 289	447 214
6X	77 396	1 182 641
8X	14 512	729 285

TABLEAU 11

RAPPORT DE CHARGE Lipopolyamine C/ADN	SANS COMPOSE (RLU)	AVEC PR2 (RLU)
4X	12 037	6 304
6X	901	113 113
8X	328	294 743

TABLEAU 12

Plasmide		Peptide		Polyethylene imine		Résultat, RLU/tumeur		Nbre d'animaux traités
quantité injectée	[ADN] $\mu\text{g}/\mu\text{l}$	référence	pept/ADN w/w	taille	Eq	moyenne	écart-type	
20	2			800 K	9	0	0	5
20	2					54 350	52 989	5
50	2			800 K	9	7 783	16 803	6
50	2	H 1	1	800 K	9	62 230	71 462	5
50	2			800 K	12	6 733	16 493	6
50	2	H 1	1	800 K	12	72 700	150 300	5
40	2			800 K	18	470	1 051	5
40	2	H 1	1	800 K	18	82 608	104 443	6
40	2			800 K	24	1 630	3 645	5
40	2	H 1	1	800 K	24	45 750	63 942	5
10	0,5	H 1	1,5	50 K lactose	12	14 152	16 946	11
10	0,5	H 1	1,5	50 K maltose	12	12 942	22 853	11

TABLEAU 10

Plasmide		Peptide		Lipide Cationique		Résultat, RLU /tumeur		Nbre d'animaux traités
µg/ tumeur	[ADN] µg/µl	référence	pept/AD N w/w	référence	nmol/ µg ADN	moyenne	écart-type	
20	2					0	0	5
20	2			A	1,8	142 300	121 418	5
20	2	H	1	"	1,8	301 730	243 166	5
30	2			"	3	99 775	128 726	6
30	2	H	1	"	3	1 340 460	1 771 624	5
7,5	0,5			A	3	88 712	49 314	5
7,5	0,5	H	1	"	3	383 313	234 713	6
7,5	0,5	H	1,5	"	3	618 025	530 774	6
15	1	II	1	"	3	1 017 372	966 141	5
10	0,5	H	1,5	C	3	679 258	414 286	9
10	0,5	H	1,5	D	3	395 433	219 333	10
18,75	0,25			C	2	222 700	126 036	6
18,75	0,25	Pr 2	0,5	"	2	1 046 050	612 401	6
20	0,5			C	4	806 467	887 206	6
20	0,5	H	1	C	4	1 348 233	1 674 106	6

TABLEAU 9

Deux jours après l'injection, on prélève le tissu tumoral qui est pesé puis haché et broyé dans 500  $\mu$ l tampon de lyse (Promega Cell Lysis Buffer E153 A). Après centrifugation (20000 g pendant 10 minutes), on prélève 10  $\mu$ l qui servent à l'évaluation de l'activité luciférase par mesure de l'émission lumineuse totale obtenue après mélange avec 50  $\mu$ l de réactif (Promega Luciferase Assay Substrate) dans un luminomètre Lumat LB 9501 (Berthold), intégration sur 10 secondes.

L'activité résultante est exprimée en RLU (Relative Lights Units) estimés dans la totalité du surnageant de lyse tumorale, ou en RLU par  $\mu$ g d'ADN injecté.

Le tableau 9 rend compte de résultats obtenus en présence de diverses lipopolyamines A, B, C ou D et le tableau 10 en présence de PEI.

Pour ce faire, 40  $\mu$ l (20  $\mu$ g d'ADN) sont injectés, en présence d'HEPES à pH 7,4 5 mM final dans la solution, puis ajout de la lipopolyamine C(RPR 120 535) dans un rapport 0,01 nmol/ $\mu$ l d'ADN 20 minutes avant injection:

Peptide, Rapport peptide / ADN poids/poids	Moyenne RLU	écart type RLU	nombre d'animaux
sans peptide	15 228 125	14 618 681	6
nls-H, 0,025 poids/poids +lipopolyamine C	42 722 500	66 485 348	6

5

TABLEAU 8

Ces résultats confirment l'effet bénéfique de l'association d'une lipopolyamine à un agent compactant selon l'invention sur la transfection in vivo dans le muscle d'un acide nucléique.

#### EXEMPLE 6

##### 10 Transfert in vivo de compositions revendiquées dans des cellules tumorales

Les essais correspondants sont effectuées sur des souris C57/BL 6 adultes (>8 semaines) femelles portant des tumeurs de type 3LL (Lewis Lung carcinoma) obtenues par passage de fragments de tumeur d'animal à animal, implantés sous la peau au niveau du flanc.

- 15 En ce qui concerne les solutions injectées elles sont préparées comme suit: L'ADN est d'abord solubilisé dans le tampon, le peptide est alors éventuellement ajouté, et après 20 minutes une solution de lipides cationiques à forte concentration (20 ou 40 mM) est ajoutée au mélange. Après addition de tous les produits, le mélange contient, outre l'ADN, le peptide et le lipide cationique, NaCl 150mM, D-Glucose 5 % et MES 5mM
- 20 pH 6,2. Dans le cas des deux dernières séries avec la lipopolyamine C (RPR 120 535), le véhicule d'injection est NaCl 75 mM et NaCl 150mM, D-glucose 5 %, respectivement.

L'injection est réalisée 20 à 30 minutes après la préparation de la solution.

- 25 On réalise l'injection de chaque composition transfectante (voir tableaux 9 et 10 pour leurs spécificités respectives) dans la tumeur 7 jours après implantation, la souris étant anesthésiée avec un mélange Kétamine 130 mg/kg+ Xylazine (4 mg/mg).

PEI 800K	Sans composé .10 <sup>6</sup> .R.L.U	H .10 <sup>6</sup> .R.L.U			nls-H .10 <sup>6</sup> .R.L.U		
Equivalent d'amines de polymère par phosphate de l'ADN	Rapport composé/ADN						
	0	0,5	1	2	0,5	1	2
6	7,7	4,9	7	12,3	4	7,3	8,8
9	5	8	11,6	4	16,3	11	12,1
12	7,5	11,3	17,1	1,2			

TABLEAU 7

**EXEMPLE 5**Transfert in vivo dans des cellules de muscles.

- 5 Les essais correspondants sont réalisés en mettant en oeuvre les matériels et protocoles suivants:
- Modèle: L'injection se pratique dans le muscle tibial cranial de souris C57 bl6 ou OF1 adultes (âgées de plus de 8 semaines)
- Protocole: L'ADN est dilué à 0,5 mg/ml dans une solution qui contiendra en final NaCl 150mM, D-Glucose 5 %. Dans certains groupes, avant injection, du peptide en solution à 1 mg/ml dans l'eau est ajouté à l'ADN en quantité suffisante pour atteindre les rapports poids/poids indiqués. Une incubation d'au moins 20 minutes à température ambiante est réalisée avant de procéder à l'injection.
- Détermination des résultats: Deux jours après l'injection, le muscle est prélevé, haché dans 750 µl de tampon de lyse (Promega E153A) additionné d'aprotinine (Sigma). Le prélèvement est homogénéisé dans un broyeur (Heidolf), et 10 µl servent à la mesure de l'activité luciférase. Cette mesure est effectuée avec un luminomètre Lumat 9501 (Berthold), en totalisant l'émission émise pendant 10 secondes après addition de 50 µl de substrat luciférase (Promega) aux 10 µl de l'échantillon. Le bruit de fond mesuré avant addition de substrat est soustrait à ce total, et l'activité est exprimé en RLU (relative light units) totaux (rapportés aux 750 ml de tampon de lyse).



- du (Dioctadécyl-carbamoylméthoxy)-acétate de 1,3-bis-(3-amino-propyl-amino)-2 propyle dans les rapport de charge indiqués.

- 2.- 5.10<sup>4</sup> cellules des lignées NIH3T3 sont incubées avec le mélange précédent à 37°C, sous une atmosphère de CO<sub>2</sub> à 5 % pendant 4 heures. Les cellules sont ensuite lavées et remises en culture pendant 48 heures dans un milieu contenant 10 % de sérum (DMEM 10 %SVF). Le tapis cellulaire est ensuite lysé, 45 heures après la transfection, dans 100 µl de tampon lyse (Promega), récupéré puis centrifugé à 20000 g pendant 5 minutes. L'activité luciférase est mesurée sur 10 µl du surnageant en ajoutant 50 µl de substrat (Promega). La lecture se fait sur luminomètre Berthold lumamat 9501® en cumulant les RLU (relative lights units) sur 10 secondes. Les résultats figurent dans le tableau 6 ci-après.

LIPOFECTANT	sans composé .10 <sup>6</sup> .R.L.U	avec H .10 <sup>6</sup> .R.L.U			avec nls-H .10 <sup>6</sup> .R.L.U		
Rapport NH <sub>2</sub> du lipofectant / phosphates de l'ADN	Rapport composé/ADN						
	0	0,5	1	2	0,5	1	2
1,8X	3,9	30,4	32,7	4,1	52,4	58,5	39,3
3X	8,9	41,5	61,3	16,6	64,1	60,4	49,2
6X	6,2	23,6	22,3	15,6			

TABLEAU 6

#### 4.2: En présence du PEI 800K

1. On procède selon le protocole décrit en 4.1 et dans un même milieu, en utilisant à titre de lipofectant le PEI 800K. Les résultats figurent dans le tableau 7 ci-après.

PEP/ADN w/v	avec H (R.L.U)	avec H-nls (R.L.U)	avec N (R.L.U)
0	2 150	2 150	2 150
0,25	3 300	38 000	35 000
0,5	45 000	100 000	35 000
1	84 000	105 000	13 000
2	105 000	200 000	20 000

TABLEAU 4

3.2: En présence de DOGS/DOPE 1,8X

On procède selon le protocole décrit précédemment en 3.1 mais en utilisant à titre d'agent de transfection une solution de 40mM de dioctadécylamidoglycyl spermine (DOGS) 1,8X en présence de dioléoylphosphatidyléthanolamine (DOPE) avec DOGS/DOPE égal à 1/2, préparée selon le mode opératoire présenté en exemple 2.

Le tableau 5 rend compte des résultats observés.

COMPOSE /ADN w/v	H (R.L.U)	H-nls (R.L.U)	N (R.L.U)
0	580	580	580
0,25	13 000	5 600	7 600
0,5	11 000	18 500	1 800
1	14 100	36 000	13 600
2	16 500	58 000	14 100

TABLEAU 5

10 EXEMPLE 4 :

Variation du rapport composé selon l'invention/ADN au sein d'une composition transfectante selon l'invention en utilisant un agent de transfection autre que le DOGS.

3.1: En présence du (Dioctadécyl-carbamoylméthoxy)-acétate de 1,3-bis-(3-amino-propylamino)-2 propyle( lipopolyamine A)

- 15 1. On prépare un mélange de 10µl composé de:
- 0,55µg d'ADN plasmidique pCMV-luc, et d'un composé selon l'invention dans le rapport indiqué, dans une solution tampon NaCl 150mM,

Rapport de charge DOGS/ADN	sans composé (R.L.U)	avec H (R.L.U)	avec chimère nls-H (R.L.U)
0,8 X D/D	24	3175	760
1 X D/D	19	7410	2380
1,8 X D/D	1800	39500	52800

TABLEAU 3

Ces résultats confirment ceux observés en exemple 1. En présence d'un composé basique selon l'invention et plus particulièrement de H, il est possible de réduire de moitié la quantité en lipofectant .

### 5      EXEMPLE 3 :

Variation du rapport composé selon l'invention/ADN au sein d'une composition transfectante selon l'invention.

#### 3.1: En présence d'une composition DOGS

1. On prépare un mélange de 10µl composé de:

10      - 0,75µg d'ADN plasmidique pCMV-luc, et d'un composé selon l'invention dans le rapport indiqué, dans une solution tampon D-glucose 5 %, NaCl 150mM, avec HEPES 10mM (pH de la solution de compaction : 7,2),

- du dioctadécylamidoglycyl spermine (DOGS) 40mM dans un rapport de charge 1,8X.

15      2.- 4.10<sup>5</sup> cellules des lignées NIH3T3 sont incubées avec le mélange précédent à 37°C, sous une atmosphère de CO<sub>2</sub> à 5 % pendant 4 heures. Les cellules sont ensuite lavées et remises en culture pendant 48 heures dans un milieu contenant 10 % de sérum (DMEM 10 %SVF). Le tapis cellulaire est ensuite lysé, 45 heures après la transfection, dans 100 µl de tampon lyse (Promega), récupéré puis centrifugé à

20      20000g pendant 5 minutes. L'activité luciférase est mesurée sur 5 µl du surnageant en ajoutant 25 µl de substrat (Promega). La lecture se fait sur luminomètre LKB en cumulant les RLU (relative lights units) sur 20 secondes.

Les résultats figurent dans le tableau 4 ci-après.

4. Compaction dans D-glucose 5 %, NaCl 150mM, avec HEPES 10mM (pH de la solution de compaction: 7,2)

Rapport de charge DOGS/ADN	sans composé (R.L.U)	avec H (R.L.U)	avec N (R.L.U)
0,8 X	880	44000	1450
1,8 X	6600	156500	-
3 X	22000	297500	90300

TABLEAU 2

- 5 Dans l'ensemble des essais, on note des résultats supérieurs lorsque la composition compactante associée à DOGS un composé selon l'invention. Il s'avère possible de diminuer de manière importante la quantité en DOGS sans porter préjudice à la capacité de transfection de la composition correspondante.

#### EXEMPLE 2 :

##### Essais de transfection en présence d'un lipide neutre

- 10 On prépare un mélange de 10µl composé comme suit:

- 0,5µg d'ADN plasmidique pCMV-luc, 0,25µg d'un composé selon l'invention, dans une solution tampon NaCl 150mM, tampon phosphate 10mM pH 7,4,

- 15 - 40mM de dioctadécylamidoglycyl spermine (DOGS) dans des rapports de charges X tels qu'indiqués dans chacun des essais en présence de dioléoylphosphatidyléthanolamine (DOPE) avec DOGS/DOPE égal à 1/2.

Dans ce cas particulier, une solution éthanolique de DOGS à 40 mM est mélangée avec un volume égal d'une solution de dioléoylphosphatidyléthanolamine (DOPE) à 80 mM, préparée dans un mélange chloroforme/éthanol (1/5). Ainsi pour un équivalent de DOGS la composition contient deux équivalents de DOPE.

- 20 10<sup>5</sup> cellules des lignées NIH3T3 sont incubées avec ce mélange dans les conditions décrites dans l'exemple précédent. A l'issue de l'incubation, ces cellules sont traitées selon le protocole de ce même exemple. Les résultats sont présentés dans le tableau ci-après.

Cet exemple décrit le transfert d'acides nucléiques in vitro (sur cultures cellulaires) au moyen d'une composition selon l'invention comprenant l'acide nucléique, un composé selon l'invention, et une lipopolyamine dans différentes conditions de pH et de tampon.

5 1. On prépare un mélange de 10 $\mu$ l composé comme suit:

- 0,5 $\mu$ g d'ADN plasmidique pCMV-luc, 0,25 $\mu$ g d'un composé selon l'invention, dans une solution tampon telle qu'identifiée,

- du dioctadécylamidoglycyl spermine (DOGS) 40mM dans des rapports de charges X tels qu'indiqués dans chacun des essais.

10 2- 5.10<sup>4</sup> cellules des lignées NIH3T3 sont incubées avec le mélange précédent à 37°C, sous une atmosphère de CO<sub>2</sub> à 5 % pendant 4 heures. Les cellules sont ensuite lavées et remises en culture pendant 48 heures dans un milieu contenant 10 % de sérum (DMEM 10 %SVF).

15 Le tapis cellulaire est ensuite lysé dans 50 $\mu$ l de tampon lyse (Promega), récupéré puis centrifugé à 20 000g pendant 5 minutes.

L'activité luciférase est mesurée sur 4 $\mu$ l du surnageant en ajoutant 20 $\mu$ l de substrat (Promega). La lecture se fait sur luminomètre LKB en cumulant les RLU (relative lights units) sur 20 secondes.

Les résultats sont présentés dans les tableaux 1 et 2 ci-après.

20 3. Compaction dans NaCl 150mM, HEPES\* (\* N-(2-hydroxyéthyl)pipérazine-N'-(acide2-éthyl-sulfonique) 10mM à pH 7,1

Rapport de charge DOGS/ADN	sans composé (R.L.U)	avec H (R.L.U)
0,8 X	32	850
1,8 X	220	23700
3 X	5400	21750

TABEAU 1

Arg(Tos)Pam ( 0,48meq/g).. Le procédé de déprotection et couplage mis en oeuvre est le suivant:

- |    |   |           |
|----|---|-----------|
|    | 1- 55 % TFA dans du dichlorométhane (DCM)   | 1 x 2 mn  |
|    | 2- 55 % TFA dans du dichlorométhane   | 1 x 30 mn |
| 5  | 3- DCM  | 2 x 1mn   |
|    | 4- DMF  | 3 x 1 mn  |
|    | 5- Couplage   |           |
|    | 6- DMF  | 2 x 1mn   |
|    | 7- DCM  | 2 x 1 mn  |
| 10 | Pour chaque étape, on utilise 10ml de solvant par gramme de résine peptide mis en oeuvre. Le couplage de tous les acides aminés ( en triple excès) est effectué dans le DMF en présence de BOP, Hobt et DIEA. Chaque étape de couplage est contrôlée par le test ninhydride |           |

- 15 Le peptide final est récupéré de la résine et entièrement déprotégé avec de l'acide fluorhydrique liquide. On utilise 10 ml de HF par gramme de peptide résine à 0°C pendant 45 minutes en présence de para-crésol et d'éthanedithiol. Après évaporation de l'acide fluorhydrique le mélange réactionnel brut est lavé avec de l'éther, dissous dans de l'acide trifluoroacétique, précipité à l'éther et séché.

### 3. Agents lipofectants mis en oeuvre

- 20 Lipopolyamine A:  
(Diocadécyl-carbamoylméthoxy)-acétate d1,3-bis-(3-amino-propylamino)-2 propyle (RP115335)

- Lipopolyamine B:  
25  $\{H_2N(CH_2)_3\}_2N(CH_2)_4N\{(CH_2)_3NH_2\}(CH_2)_3NHCH_2COGlyN[(CH_2)_{17}-CH_3]_2$   
(RP120525)

- Lipopolyamine C:  
 $H_2N(CH_2)_3NH(CH_2)_4NH(CH_2)_3NHCH_2COGlyN[(CH_2)_{18}]_2$  (RP120535)

- Lipopolyamine D:  
 $H_2N(CH_2)_3NH(CH_2)_4NH(CH_2)_3NHCH_2COArgN[(CH_2)_{18}]_2$  (RP120531)

- 30 EXEMPLE 1:

### Transfert d'acide nucléique in vitro dans des cellules NIH 3T3

2.1 N: ATPAKKAAATPAKKAA(COOH)

Cet oligopeptide a été synthétisé sous forme de sel d'acide trifluoroacétique au moyen d'un synthétiseur de peptides Applied Biosystem 431A, sur une résine HMP (Applied Biosystem) et selon une stratégie FMOC. Après la synthèse, le peptide a été libéré de la résine par traitement 90 minutes en présence d'une solution eau/TFA 1/19. Après filtration, la solution est concentrée sur évaporateur rotatif, puis le peptide est précipité 2 fois par addition d'éther tertibutylméthylique à partir de solution dans le TFA. Le culot final est lavé par l'éther tertibutylméthylique puis séché. Le peptide est solubilisé dans 5 ml d'eau, filtré et purifié par HPLC phase inverse sur colonne C18 100 Å (Biorad RSL). le peptide est purifié par à l'aide d'un gradient de 0 à 25% d'acétonitrile, 0,07% TFA dans l'eau 0,07% TFA. La pureté du peptide obtenu est supérieure à 95% et sa solubilité dans l'eau de 100 mg/ml.

2.2 nls: PKKKRKV

Cet oligopeptide a été synthétisé sous forme de sel d'acide trifluoroacétique selon le protocole décrit ci-dessus en utilisant pour le clivage une solution eau/TFA/phénol/éthanedithiol/thioanisole 2/ 40/3/1/2 (v/v). La pureté du peptide obtenu est supérieure à 95% et sa solubilité dans l'eau de 100 mg/ml à pH 2,1.

2.3 H: KTPKKAKKPKTKKAKKP(COOH) et nls-H: PKKKRKV-bAla-KTPKKAKKPKTKKAKKP(COOH)

Ces oligopeptides sont synthétisés sous forme de sel d'acide trifluoroacétique selon le protocole décrit ci-dessus. Pour ce faire, on divise la résine en deux lots. Un premier lot destiné à la synthèse de H est traité pour le clivage avec une solution TFA/phénol/éthanedithiol/thioanisole/eau 40/3/1/2/2 (v/v). La pureté du peptide obtenu est supérieure à 90% et sa solubilité dans l'eau de 10 mg/ml à pH 2,1. Sur le second lot de résine la synthèse est poursuivie de manière à obtenir nls-bAla-H. La solution de clivage mise en oeuvre est identique à la précédente. La pureté du peptide obtenu est supérieure à 95 % et sa solubilité dans l'eau de 10 mg/ml à pH 2,1.

2.4 PR1 SRSRYRQRQRSSRRRRR et PR2: RRRLHRIHRROHRSCRRRKRR

Ces oligopeptides sont assemblés en plusieurs étapes, en synthèse phase solide selon la technique BocBenzyle. La résine de départ est une résine Boc-L-

ATPKKSAKKTPKKAKKP(COOH),  
 KKAQSPKKAKAAKPKKAPKSPAKAKA(COOH), SRSRYRQRQSRRRRRR  
 (COOH) et RRRLHRIHRRQHRSCRRRKRR(COOH).

- 5 pour effectuer le transfert in vitro, ex vivo et/ou in vivo d'au moins un acide nucléique,  
 ledit oligonucléotide étant associé ou non à un élément de ciblage.

La présente invention sera plus complètement décrite à l'aide des exemples qui suivent, qui doivent être considérés comme illustratifs et non limitatifs.

#### Matériel et Méthodes :

##### 1. Plasmides utilisés pour le transfert de gènes in vivo

- 10 Les constructions utilisées pour mettre en évidence l'activité des compositions de l'invention sont des plasmides comportant le gène codant pour la luciférase (Luc)

Il s'agit notamment de plasmides pCMV luc, pXL 2621, pXL 2622, qui tous contiennent le gène codant pour la luciférase (tiré du vecteur pGL2, Promega) en aval  
 15 du promoteur du cytomegalovirus (CMV) extrait de pCDNA3 (Invitrogen). pCMV luc et pXL2622 dérivent d'un vecteur pGL2, pXL 2621 d'un vecteur pGL2 contrôle, et dans tous ces vecteurs le promoteur SV40 a été remplacé par le promoteur CMV.

De manière générale les plasmides sont obtenus par le technique de précipitation au PEG (Ausubel), et stockés dans du Tris 10mM EDTA 1mM pH 8 à 4  
 20 °C à une concentration d'environ 10 µg d'ADN par µl.

##### 2. Composés utilisés selon l'invention:

H: KTPKKAKKPKTPKKAKKP(COOH) 18 AA

N: ATPAKKAAATPAKKAA(COOH) 16 AA

nls-H: PKKKRKV-bAla-KTPKKAKKPKTKKAKKP(COOH) 26 AA

25 PR1: RRRLHRIHRRQHRSCRRRKRR 21 AA

PR2: SRSRYRQRQSRRRRRR

Ils ont été préparés comme suit:



stériles, isotoniques, ou de compositions sèches, notamment lyophilisées, qui, par addition selon le cas d'eau stérilisée ou de sérum physiologique, permettent la constitution de solutés injectables. Les doses d'acide nucléique utilisées pour l'injection ainsi que le nombre d'administrations peuvent être adaptées en fonction de différents  
5 paramètres, et notamment en fonction du mode d'administration utilisé, de la pathologie concernée, du gène à exprimer, ou encore de la durée du traitement recherchée.

Elles peuvent être avantageusement utilisées pour transfecter une grande variété de type cellulaire comme par exemple les cellules hématopoïétiques, les  
10 lymphocytes, les hépatocytes, les cellules endothéliales, les cellules de mélanomes, de carcinomes et de sarcomes, les cellules musculaires lisses, les neurones et les astrocytes.

La présente invention fournit ainsi une méthode particulièrement avantageuse pour le traitement de maladies utilisant la transfection in vitro, ex vivo ou in vivo d'un  
15 acide nucléique apte à corriger ladite maladie en association avec un agent de transfection de type polymère cationique ou lipofectant, et un composé tel que défini ci-avant. Plus particulièrement, cette méthode est applicable aux maladies résultant d'une déficience en un produit protéique ou nucléique et l'acide nucléique administré code pour ledit produit protéique ou contient la séquence correspondant audit produit  
20 nucléique. Les compositions selon l'invention sont particulièrement intéressantes pour leur biodisponibilité et leur haut niveau de transfection.

La présente invention concerne également toute utilisation d'un composé constitué en tout ou partie, de motifs peptidiques (KTPKKAKKP) et/ou (ATPAKKAA) avec le nombre de ces motifs pouvant varier entre 2 et 10, pour,  
25 lorsqu'ils sont couplés à un ligand de récepteur cellulaire, un anticorps ou dérivé d'anticorps, cibler un acide nucléique vers des cellules exprimant les récepteurs ou anti-gènes correspondants. Dans cette perspective, un ligand, anticorps ou dérivé d'anticorps potentiel est couplé audit composé et l'on apprécie le pouvoir de transfection de cette molécule chimère comparativement au composé seul.

30 La présente invention couvre également toute utilisation d'un oligopeptide sélectionné parmi:

(ATPAKKAA)<sub>2</sub>(COOH),

(KTPKKAKKP)<sub>2</sub>(COOH),

montré que l'addition d'un lipide neutre permet d'améliorer la formation des particules nucléolipidiques et, de manière surprenante, de favoriser la pénétration de la particule dans la cellule en déstabilisant sa membrane.

5 Plus préférentiellement, les lipides neutres utilisés dans le cadre de la présente invention sont des lipides à 2 chaînes grasses.

De manière particulièrement avantageuse, on utilise des lipides naturels ou synthétiques, zwitterioniques ou dépourvus de charge ionique dans les conditions physiologique. Il peuvent être choisis plus particulièrement parmi la dioleoylphosphatidyléthanolamine (DOPE), le oléoyl-palmitoylphosphatidyléthanolamine (POPE), le  
10 di-stéaroyl-, -palmitoyl-, -mirystoyl phosphatidyléthanolamine ainsi que leurs dérivé N-méthylés 1 à 3 fois; les phosphatidylglycérols, les diacylglycérols, les glycosyldiacylglycérols, les cérébrosides (tels que notamment les galactocérébrosides), les sphingolipides (tels que notamment les sphingomyélines) ou encore les asialogangliosides (tels que notamment les asialoGM1 et GM2).

15 Ces différents lipides peuvent être obtenus soit par synthèse, soit par extraction à partir d'organes (exemple : le cerveau) ou d'oeufs, par des techniques classiques bien connues de l'homme du métier. En particulier, l'extraction des lipides naturels peut être réalisée au moyen de solvants organiques (voir également Lehninger, Biochemistry).

Préférentiellement, les compositions de l'invention, mettant en oeuvre à titre  
20 d'agent de transfection un lipofectant, comprennent de 0,1 à 20 équivalents de lipide neutre pour 1 équivalent de lipopolyamine, et, plus préférentiellement, de 1 à 5. Dans le cas où l'agent de transfection est un polymère cationique, les compositions de l'invention comprennent, en plus du polymère cationique dans les rapports cités ci-avant, de 0,1 à 20 équivalents molaires de lipide neutre pour 1 équivalent molaire de  
25 phosphate de l'acide nucléique, et, plus préférentiellement, de 1 à 5.

Les compositions selon l'invention peuvent être formulées en vue d'administrations par voie topique, cutanée, orale, rectale, vaginale, parentérale, intranasale, intraveineuse, intramusculaire, sous-cutanée, intraoculaire, transdermique, etc... De préférence, les compositions pharmaceutiques de l'invention contiennent un  
30 véhicule pharmaceutiquement acceptable pour une formulation injectable, notamment pour une injection directe au niveau de l'organe désiré, ou pour une administration par voie topique (sur peau et/ou muqueuse). Il peut s'agir en particulier de solutions

bloquer ainsi leur traduction en protéine, selon la technique décrite dans le brevet EP 140 308. Les antisens comprennent également les séquences codant pour des ribozymes, qui sont capables de détruire sélectivement des ARN cibles (EP 321 201).

5 Comme indiqué plus haut, l'acide nucléique peut également comporter un ou plusieurs gènes codant pour un peptide antigénique, capable de générer chez l'homme ou l'animal une réponse immunitaire. Dans ce mode particulier de mise en oeuvre, l'invention permet donc la réalisation soit de vaccins soit de traitements  
10 immunothérapeutiques appliqués à l'homme ou à l'animal, notamment contre des microorganismes, des virus ou des cancers. Il peut s'agir notamment de peptides antigéniques spécifiques du virus d'Epstein Barr, du virus HIV, du virus de l'hépatite B (EP 185 573), du virus de la pseudo-rage, ou encore spécifiques de tumeurs (EP 259 212).

Préférentiellement, l'acide nucléique comprend également des séquences permettant l'expression du gène thérapeutique et/ou du gène codant pour le peptide  
15 antigénique dans la cellule ou l'organe désiré. Il peut s'agir des séquences qui sont naturellement responsables de l'expression du gène considéré lorsque ces séquences sont susceptibles de fonctionner dans la cellule infectée. Il peut également s'agir de séquences d'origine différente (responsables de l'expression d'autres protéines, ou même synthétiques). Notamment, il peut s'agir de séquences promotrices de gènes  
20 eucaryotes ou viraux. Par exemple, il peut s'agir de séquences promotrices issues du génome de la cellule que l'on désire infecter. De même, il peut s'agir de séquences promotrices issues du génome d'un virus. A cet égard, on peut citer par exemple les promoteurs des gènes E1A, MLP, CMV, RSV, etc. En outre, ces séquences d'expression peuvent être modifiées par addition de séquences d'activation, de  
25 régulation, etc.

Par ailleurs, l'acide nucléique peut également comporter, en particulier en amont du gène thérapeutique, une séquence signal dirigeant le produit thérapeutique synthétisé dans les voies de sécrétion de la cellule cible. Cette séquence signal peut  
30 être la séquence signal naturelle du produit thérapeutique, mais il peut également s'agir de toute autre séquence signal fonctionnelle, ou d'une séquence signal artificielle.

Plus préférentiellement, les compositions de l'invention comprennent en outre, un ou plusieurs lipides neutres. De telles compositions sont particulièrement avantageuses, notamment lorsque le rapport R est faible. La demanderesse a en effet

ainsi codé peut être une protéine, un peptide, etc. Ce produit protéique peut être  
homologue vis-à-vis de la cellule cible (c'est-à-dire un produit qui est normalement  
exprimé dans la cellule cible lorsque celle-ci ne présente aucune pathologie). Dans ce  
cas, l'expression d'une protéine permet par exemple de pallier une expression  
5 insuffisante dans la cellule ou l'expression d'une protéine inactive ou faiblement active  
en raison d'une modification, ou encore de surexprimer ladite protéine. Le gène  
thérapeutique peut aussi coder pour un mutant d'une protéine cellulaire, ayant une  
stabilité accrue, une activité modifiée, etc. Le produit protéique peut également être  
hétérologue vis-à-vis de la cellule cible. Dans ce cas, une protéine exprimée peut par  
10 exemple compléter ou apporter une activité déficiente dans la cellule, lui permettant de  
lutter contre une pathologie, ou stimuler une réponse immunitaire.

Parmi les produits thérapeutiques au sens de la présente invention, on peut  
citer plus particulièrement les enzymes, les dérivés sanguins, les hormones, les  
lymphokines : interleukines, interférons, TNF, etc (FR 9203120), les facteurs de  
15 croissance, les neurotransmetteurs ou leurs précurseurs ou enzymes de synthèse, les  
facteurs trophiques : BDNF, CNTF, NGF, IGF, GMF, aFGF, bFGF, VEGF, NT3,  
NT5, HARP/pléiotrophine, les gènes correspondant aux protéines impliquées dans le  
métabolisme des lipides, de type apolipoprotéine choisie parmi les apolipoprotéines A-  
I, A-II, A-IV, B, C-I, C-II, C-III, D, E, F, G, H, J et apo(a), les enzymes du  
20 métabolisme comme par exemple la lipoprotéine lipase, la lipase hépatique, la lécithine  
cholestérol acyltransférase, la 7 alpha cholestérol hydroxylase, la phosphatidique acide  
phosphatase, ou encore des protéines de transfert de lipides comme la protéine de  
transfert des esters de cholestérol et la protéine de transfert des phospholipides, une  
protéine de liaisons des HDL ou encore un récepteur choisi par exemple parmi les  
25 récepteurs LDL, récepteurs des chylomicrons-remnants et les récepteurs scavenger, la  
dystrophine ou une minidystrophine (FR 9111947), la protéine GAX, la protéine  
CFTR associée à la mucoviscidose, les gènes suppresseurs de tumeurs : p53, Rb,  
Rap1A, DCC, k-rev, etc (FR 93 04745), les gènes codant pour des facteurs impliqués  
dans la coagulation : Facteurs VII, VIII, IX, les gènes intervenant dans la réparation  
30 de l'ADN, les gènes suicides (thymidine kinase, cytosine déaminase), etc.

Le gène thérapeutique peut également être un gène ou une séquence antisens,  
dont l'expression dans la cellule cible permet de contrôler l'expression de gènes ou la  
transcription d'ARNm cellulaires. De telles séquences peuvent, par exemple, être  
transcrites dans la cellule cible en ARN complémentaires d'ARNm cellulaires et

le  $\text{H}_2\text{N}(\text{CH}_2)_3\text{NH}(\text{CH}_2)_4\text{NH}(\text{CH}_2)_3\text{NHCH}_2\text{COGlyN}[(\text{CH}_2)_{18}]_2$  ou le  
 $\text{H}_2\text{N}(\text{CH}_2)_3\text{NH}(\text{CH}_2)_4\text{NH}(\text{CH}_2)_3\text{NHCH}_2\text{COArgN}[(\text{CH}_2)_{18}]$ .

Pour obtenir un effet optimum des compositions de l'invention, les proportions respectives de la polyamine et de l'acide nucléique sont de préférence déterminées de sorte que le rapport R charges positives de l'agent de transfection/ charges négatives de l'acide nucléique soit compris entre 0,1 et 10 et plus préférentiellement entre 0,5 et 6.

La présence d'un composé selon l'invention au sein d'une composition transfectante permet avantageusement de diminuer considérablement la quantité en agent de transfection. Il s'en suit une toxicité nettement amoindrie qui rend désormais possible par exemple la transfection de cellules sensibles à l'origine à l'agent de transfection comme par exemple les cellules hématopoïétiques avec les lipopolyamines. Enfin, comme le démontrent les exemples ci-après, le pouvoir transfectant des compositions selon l'invention est supérieur à celui obtenu avec les compositions transfectantes classiques.

Dans les compositions de la présente invention, l'acide nucléique peut être aussi bien un acide désoxyribonucléique qu'un acide ribonucléique. Il peut s'agir de séquences d'origine naturelle ou artificielle, et notamment d'ADN génomique, d'ADNc, d'ARNm, d'ARNt, d'ARNr, de séquences hybrides ou de séquences synthétiques ou semi-synthétiques. Ces acides nucléiques peuvent être d'origine humaine, animale, végétale, bactérienne, virale, etc. Ils peuvent être obtenus par toute technique connue de l'homme du métier, et notamment par criblage de banques, par synthèse chimique, ou encore par des méthodes mixtes incluant la modification chimique ou enzymatique de séquences obtenues par criblage de banques. Ils peuvent par ailleurs être incorporés dans des vecteurs, tels que des vecteurs plasmidiques.

Concernant plus particulièrement les acides désoxyribonucléiques, ils peuvent être simple ou double brin. Ces acides désoxyribonucléiques peuvent coder pour des gènes thérapeutiques, des séquences régulatrices de la transcription ou de la réplication, des séquences antisens, des régions de liaison à d'autres composants cellulaires, etc.

Au sens de l'invention, on entend par gène thérapeutique notamment tout gène codant pour un produit protéique ayant un effet thérapeutique. Le produit protéique

- R<sub>3</sub>, R<sub>4</sub> et R<sub>5</sub> représentant indépendamment l'un de l'autre un atome d'hydrogène ou un radical alkyle, substitué ou non, en C<sub>1</sub> à C<sub>4</sub>, avec p pouvant varier entre 0 et 5,

5 - R<sub>6</sub> représentant un dérivé du cholestérol ou un groupement alkyle amino - NR<sub>1</sub>R<sub>2</sub> avec R<sub>1</sub> et R<sub>2</sub> représentant indépendamment l'un de l'autre un radical aliphatique, saturé ou non, linéaire ou ramifié en C<sub>12</sub> à C<sub>22</sub>.

A titre représentatif de ces lipopolyamines on peut tout particulièrement citer le (Diocadécyl-carbamoylméthoxy)-acétate de 2-5-bis-(3-amino-propylamino)-pentyle et le (Diocadécyl-carbamoylméthoxy)-acétate de 1,3-bis-(3-amino-propylamino)-2 propyle dit ci-après lipopolyamine A.

Les demandes de brevet EP 394 111 et FR 94 145 96 décrivent également un procédé utilisable pour la préparation des lipopolyamines correspondantes.

Enfin plus récemment, de nouvelles lipopolyamines, valorisables également dans le cadre de la présente invention, ont été décrites dans la demande de brevet FR 95/134 90. A titre représentatif de ces lipopolyamines on peut plus particulièrement mentionner celles qui suivent:

- lipopolyamine B:

$\{H_2N(CH_2)_3\}_2N(CH_2)_4N\{(CH_2)_3NH_2\}(CH_2)_3NHCH_2COGlyN[(CH_2)_{17}-CH_3]_2$   
(RP120525)

20 - lipopolyamine C:

$H_2N(CH_2)_3NH(CH_2)_4NH(CH_2)_3NHCH_2COGlyN[(CH_2)_{18}]_2$  (RP120535)

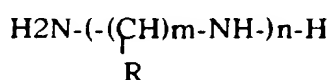
- lipopolyamine D:

$H_2N(CH_2)_3NH(CH_2)_4NH(CH_2)_3NHCH_2COArgN[(CH_2)_{18}]$  (RP120531)

25 De manière particulièrement avantageuse, on peut utiliser dans le cadre de l'invention la dioctadécylamidoglycyl spermine (DOGS) la 5-carboxyspermylamide de la palmitoylphosphatidylethanolamine (DPPES), le (Diocadécyl-carbamoylméthoxy)-acétate de 2-5-bis-(3-amino-propylamino)-pentyle, le (Diocadécyl-carbamoylméthoxy)-acétate de 1,3-bis-(3-amino-propylamino)-2 propyle, le  $\{H_2N(CH_2)_3\}_2N(CH_2)_4N\{(CH_2)_3NH_2\}(CH_2)_3NHCH_2COGlyN[(CH_2)_{17}-CH_3]_2$ .

inclusivement et n est compris entre 1 et 5 inclusivement. Encore plus  
préférentiellement, la région polyamine est représentée par la spermine, la thermine ou  
un de leurs analogues ayant conservé des propriétés de liaison à l'ADN. Quant à la  
région lipophile, elle est représentée par au moins une chaîne hydrocarbonée, saturée  
ou non, du cholestérol, un lipide naturel ou un lipide synthétique capable de former des  
phases lamellaires ou hexagonales, liée de manière covalente à la région hydrophile.

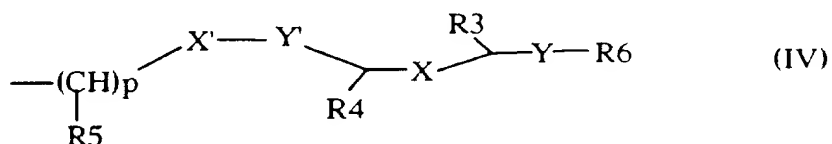
La demande de brevet EP 394 111 décrit d'autres lipopolyamines de formule générale III susceptibles d'être mises en oeuvre dans le cadre de la présente invention.



10 dans laquelle R représente notamment un radical de formule générale (R<sub>1</sub>R<sub>2</sub>)  
N-CO-CH-NH-CO-.

A titre représentatif de ces lipopolyamines on peut plus particulièrement citer la dioctadécylamidoglycyl spermine (DOGS) et la 5-carboxyspermylamide de la palmitoylphosphatidylethanolamine (DPPES).

15 Les lipopolyamines décrites dans la demande de brevet FR 94 14596 peuvent également être utilisées avantageusement à titre d'agent de transfection selon l'invention. Elles sont représentées par la formule générale IV ci-dessus dans laquelle R représente



20 avec

- X et X' représentant, indépendamment l'un de l'autre, un atome d'oxygène, un groupement méthylène  $-(CH_2)_q$ - avec q égal à 0, 1, 2 ou 3, ou un groupement amino
- NH- ou -NR'- avec R' représentant un groupement alkyle en C<sub>1</sub> à C<sub>4</sub>,
- Y et Y' représentant indépendamment l'un de l'autre un groupement méthylène, un groupement carbonyle ou un groupement C=S,

Selon un mode particulier de l'invention, l'agent lipidique mis en oeuvre possède une région cationique. Cette région cationique, préférentiellement polyamine, chargée cationiquement, est capable de s'associer de manière réversible avec l'acide nucléique, chargé négativement. Cette interaction compacte fortement l'acide nucléique. La région lipophile rend cette interaction ionique inaccessible au milieu aqueux externe, en recouvrant la particule nucléolipidique formée d'une pellicule lipidique.

On sait ainsi qu'un lipide cationique chargé positivement, le chlorure de N-[1-(2,3-dioleoyloxy)propyl]-N,N,N-triméthylammonium (DOTMA), interfère, sous la forme de liposomes ou de petites vésicules, spontanément avec de l'ADN, qui est chargé négativement, pour former des complexes lipides-ADN, capables de fusionner avec les membranes cellulaires, et permet ainsi la délivrance intracellulaire de l'ADN. Depuis le DOTMA, d'autres lipides cationiques sont proposés sur ce modèle de structure : groupe lipophile associé à un groupement amino via un bras dit "spacer". Parmi ceux-ci, on peut plus particulièrement citer ceux comprenant à titre de groupement lipophile deux acides gras ou un dérivé du cholestérol, et comportant, en outre, le cas échéant, à titre de groupement amino, un groupement d'ammonium quaternaire. Les DOTAP, DOBT ou le ChOTB peuvent notamment être cités à titre représentatifs de cette catégorie de lipides cationiques. D'autres composés, comme les DOSC et ChOSC, se caractérisent par la présence d'un groupement choline à la place du groupement d'ammonium quaternaire. Une autre catégorie de lipides cationiques, les lipopolyamines, a également été décrite. Dans ce type de composés, le groupement cationique est représenté par le radical L-5carboxyspermine qui contient quatre groupements d'ammonium, deux primaires et deux secondaires. Les DOGS et DPPES en font notamment partie. Ces lipopolyamines sont tout particulièrement efficaces pour la transfection de cellules endocrines primaires.

Avantageusement, les lipofectants convenant à l'invention peuvent également être choisis parmi des lipopolyamines dont la région polyamine répond à la formule générale (II)



dans laquelle m est un nombre entier supérieur ou égal à 1 et n est un nombre entier supérieur ou égal à 1, m pouvant varier entre les différents groupes de carbone compris entre deux amines. Préférentiellement, m est compris entre 2 et 6



étant entendu que la somme  $p+q$  est telle que le poids moléculaire moyen du polymère soit compris entre 100 et  $10^7$  Da.

Il est entendu que, dans la formule (I) la valeur de  $n$  peut varier entre les différents motifs  $p$ . Ainsi, la formule (I) regroupe à la fois les homopolymères et les  
5 hétéropolymères.

Plus préférentiellement, dans la formule (I),  $n$  est compris entre 2 et 5. En particulier, les polymères de polyéthylène imine (PEI) et polypropylène imine (PPI) présentent des propriétés tout à fait avantageuses. Les polymères préférés pour la mise en oeuvre de la présente invention sont ceux dont le poids moléculaire est compris  
10 entre  $10^3$  et  $5 \cdot 10^6$ . A titre d'exemple, on peut citer le polyéthylène imine de poids moléculaire moyen 50 000 Da (PEI50K) ou le polyéthylène imine de poids moléculaire moyen 800 000 Da (PEI800K).

Le PEI50K ou Le PEI800K sont accessibles commercialement. Quant aux autres polymères représentés par la formule générale I, ils peuvent être préparés selon  
15 le procédé décrit dans la demande de brevet FR 94 08735.

Pour obtenir un effet optimum des compositions de l'invention, les proportions respectives du polymère et de l'acide nucléique sont de préférence déterminées de sorte que le rapport molaire  $R = \text{amines du polymère} / \text{phosphates de l'acide nucléique}$  soit compris entre 0,5 et 50, plus préférentiellement entre 5 et 30. Des résultats tout  
20 particulièrement avantageux sont obtenus en utilisant de 5 à 25 équivalents d'amines de polymère par charge d'acide nucléique.

En ce qui concerne plus particulièrement les lipofectants, on entend couvrir au sens de l'invention, sous cette dénomination, tout composé à caractère lipidique et déjà proposé à titre d'agent actif à l'égard de la transfection cellulaire d'acides nucléiques.  
25 De manière générale, il s'agit de molécules amphiphiles comprenant au moins une région lipophile associée ou non à une région hydrophile. A titre représentatif de la première famille de composés, on peut notamment proposer les lipides susceptibles de former des liposomes comme les POPC, phosphatidylserine, phosphatidylcholine, cholestérol, maléimidophénylbutyrylphosphatidyléthanolamine, lactosylcéramide en  
30 présence ou non de polyéthylène glycol pour former des liposomes furtifs ou, avec ou sans anticorps ou ligands, pour former des immunoliposomes ou des liposomes ciblés.

A titre illustratif de ce type d'association, on peut notamment mettre en oeuvre dans le cadre de la présente invention, un composé de type H<sub>1</sub>-nls et plus préférentiellement un peptide possédant la séquence PKKKRKV-bAla-(KTPKKAKKP)<sub>2</sub>(COOH) (SEQ ID N°9).

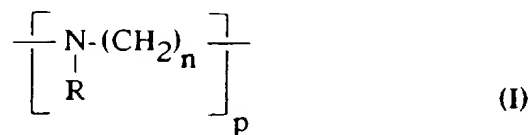
- 5      Avantageusement, le composé selon l'invention et plus particulièrement tout dérivé d'histone, de protamine ou de nucléoline peut être en outre polyglycosylé, sulfoné et/ou phosphorylé et/ou greffé à des sucres complexes ou à un composé lipophile comme par exemple une chaîne polycarbonée ou un dérivé du cholestérol.

- 10      La composition selon l'invention peut bien entendu comprendre plusieurs composés compactant l'acide nucléique, de nature différente. On peut ainsi associer un composé de type histone à un composé de type nucléoline.

- 15      Le composé selon l'invention est présent en quantité suffisante pour compacter l'acide nucléique selon l'invention. C'est ainsi que le rapport composé/acide nucléique (exprimé en poids) peut être compris entre 0,1 et 10 et plus préférentiellement entre 0,3 et 3.

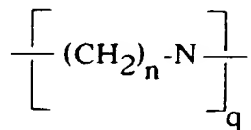
En ce qui concerne l'agent de transfection présent dans la composition selon l'invention, il est préférentiellement choisi parmi les polymères cationiques et les lipofectants.

- 20      Selon la présente invention, le polymère cationique est de préférence un composé de formule générale I,



dans laquelle

- R peut être un atome d'hydrogène ou un groupe de formule



- 25      - n est un nombre entier compris entre 2 et 10;  
       - p et q sont des nombres entiers,

Dans un mode de réalisation particulièrement avantageux, les compositions de la présente invention comprennent en outre un élément de ciblage permettant d'orienter le transfert de l'acide nucléique. Cet élément de ciblage peut être un élément de ciblage extracellulaire, permettant d'orienter le transfert de l'acide nucléique vers  
5 certains types cellulaires ou certains tissus souhaités (cellules tumorales, cellules hépatiques, cellules hématopoiétiques, etc). Il peut également s'agir d'un élément de ciblage intracellulaire, permettant d'orienter le transfert de l'acide nucléique vers certains compartiments cellulaires privilégiés (mitochondries, noyau, etc).

Plus préférentiellement, l'élément de ciblage est lié, de manière covalente ou  
10 non covalente, au composé selon l'invention. L'élément de ciblage peut également être lié à l'acide nucléique. Selon un mode privilégié de l'invention, ledit composé est associé, via une partie hétérologue supplémentaire liée à une de ses extrémités. De telles parties peuvent notamment être des peptides de type fusogène pour favoriser la transfection cellulaire c'est-à-dire privilégier le passage de la composition transfectante  
15 ou de ses divers éléments à travers des membranes, aider à la sortie des endosomes ou encore pour traverser la membrane nucléaire. Il peut également s'agir d'un ligand de récepteur cellulaire présent à la surface du type cellulaire comme par exemple un sucre, la transferrine, l'insuline ou la protéine asialo-orosomucoïde. Il peut également s'agir d'un ligand de type intracellulaire comme une séquence signal de location  
20 nucléaire, nls, qui privilégie l'accumulation de l'ADN transfecté au sein du noyau.

Parmi les éléments de ciblage utilisables dans le cadre de l'invention, on peut citer les sucres, les peptides, les hormones, les vitamines, les cytokines, les oligonucléotides, les lipides ou des séquences ou fractions issues de ces éléments et permettant une liaison spécifique avec les récepteurs correspondants.  
25 Préférentiellement, il s'agit de sucres et/ou peptides tels que des anticorps ou fragments d'anticorps, des ligands de récepteurs cellulaires ou des fragments de ceux-ci, des récepteurs ou fragments de récepteurs, etc. En particulier, il peut s'agir de ligands de récepteurs de facteurs de croissance, de récepteurs de cytokines, de récepteurs de lectines cellulaires ou de récepteurs de protéines d'adhésion. On peut  
30 également citer le récepteur de la transferrine, des HDL et des LDL. L'élément de ciblage peut également être un sucre permettant de cibler des lectines tels que les récepteurs asialoglycoprotéiques, ou encore un fragment Fab d'anticorps permettant de cibler le récepteur du fragment Fc des immunoglobulines.

ATPKKSAKKTPKKAKKP(COOH). (SEQ ID N°5) et  
KKAkSPKKAKAAKPKKAPKSPAKAKA (COOH). (SEQ ID N°6).

En ce qui concerne plus particulièrement les séquences dérivant de la  
protamines pouvant être également mises en oeuvre dans le cadre de la présente  
5 invention, on peut notamment proposer les oligopeptides suivants:  
SRSRYRQRQSRRRRRR(COOH). (SEQ ID N°7) et  
RRRLHRIHRRQHRSRRRKRR(COOH). (SEQ ID N°8).

Au sens de la présente invention, le terme dérivé désigne tout peptide,  
pseudopeptide (peptide incorporant des éléments non biochimiques) ou protéine  
10 différant de la protéine ou peptide considéré, obtenu par une ou plusieurs  
modifications de nature génétique et/ou chimique. Par modification de nature  
génétique et/ou chimique, on peut entendre toute mutation, substitution, délétion,  
addition et/ou modification d'un ou plusieurs résidus de la protéine considérée. Plus  
précisément, par modification chimique, on entend toute modification du peptide ou  
15 protéine générée par réaction chimique ou par greffage chimique de molécule(s),  
biologique(s) ou non, sur un nombre quelconque de résidus de la protéine. Par  
modification génétique, on entend toute séquence peptidique dont l'ADN hybride avec  
ces séquences ou des fragments de celles-ci et dont le produit possède l'activité  
indiquée. De tels dérivés peuvent être générés dans des buts différents, tels que  
20 notamment celui d'augmenter l'affinité du polypeptide correspondant pour son(s)  
ligand(s), celui d'améliorer ses niveaux de production, celui d'augmenter sa résistance à  
des protéases, celui d'augmenter et/ou de modifier son activité, ou celui de lui conférer  
de nouvelles propriétés pharmacocinétiques et/ou biologiques. Parmi les dérivés  
résultant d'une addition, on peut citer par exemple les séquences peptidiques chimères  
25 comportant une partie hétérologue supplémentaire liée à une extrémité. Le terme  
dérivé comprend également les séquences protéiques homologues à la séquence  
considérée, issues d'autres sources cellulaires et notamment de cellules d'origine  
humaine, ou d'autres organismes, et possédant une activité du même type. De telles  
séquences homologues peuvent être obtenues par des expériences d'hybridation de  
30 l'ADN correspondant. Les hybridations peuvent être réalisées à partir de banques  
d'acides nucléiques, en utilisant comme sonde la séquence native ou un fragment de  
celle-ci, dans des conditions de stringence conventionnelles (Maniatis et al.), (Cf  
techniques générales de biologie moléculaire), ou, de préférence, dans des conditions  
de stringence élevées.

continue ou non. C'est ainsi qu'ils peuvent être séparés par des liens de nature biochimique, par exemple un ou plusieurs acides aminés ou de nature chimique.

Le choix particulier à titre de composé selon l'invention, d'un peptide ou pseudopeptide possédant une majorité d'acides aminés à caractère basique comme la lysine, l'histidine ou l'arginine est particulièrement avantageux dans le cadre de la présente invention. Ce composé peut en outre posséder une structure conformationnelle feuillet  $\beta$ . Les acides aminés basiques sont en effet plus spécifiquement impliqués dans les liaisons peptide-acide nucléique. Ils participent à l'établissement des liaisons ioniques hydrogène entre les deux entités favorisant ainsi la condensation de l'acide nucléique. Quant à la structure feuillet  $\beta$ , elle se caractérise par une meilleure accessibilité d'une majorité des liaisons carbonyles et des atomes d'hydrogène qui de part leurs caractères accepteur et donneur respectifs privilégient également la formation de liaisons avec l'acide nucléique à compacter.

Il s'agit plus préférentiellement, de tout ou partie d'une histone, d'une nucléoline, de la protamine et/ou un de leurs dérivés.

Les histones et les protamines sont des protéines cationiques compactant naturellement l'ADN. Elles sont ainsi responsables in vivo de la condensation de l'ADN non transcrit, de l'ADN de certains virus. A titre d'histones pouvant être mises en oeuvre dans le cadre de la présente invention, on peut plus particulièrement citer les histones H1, H2a, H3 et H4. En ce qui concerne la nucléoline, il s'agit d'une protéine nucléolaire qui semble posséder un effet synergique vis à vis de l'histone H1 lors de la condensation de l'ADN par celle-ci. Dans le cadre de la présente invention, le composé peut être avantageusement représenté par une séquence peptidique dérivant de la partie N terminale de la nucléoline, et plus précisément correspondant à la séquence (ATPAKKAA)<sub>2</sub>(COOH) (SEQ ID N°3).

De préférence, le composé mis en oeuvre selon l'invention est une séquence dérivée de l'histone H1, et plus préférentiellement de son domaine C terminal et plus particulièrement correspond à la séquence (KTPKKAKKP)<sub>2</sub>(COOH). (SEQ ID N°4)

A titre illustratif de cette famille de composés selon l'invention on peut également citer les oligopeptides suivants:

Toutefois, les vecteurs synthétiques proposés aujourd'hui sont encore loin d'être aussi performants que les vecteurs viraux. Ceci pourrait être la conséquence d'une condensation insuffisante de l'ADN à transfecter et/ou des difficultés rencontrées par l'ADN transfecté pour sortir de l'endosome et pénétrer dans le noyau cellulaire.

5 Enfin, d'autres inconvénients sont directement associés à la nature des polymères cationiques des lipofectants utilisés.

La présente invention a précisément pour objectif de proposer une solution avantageuse à ces problèmes.

10 Plus précisément, la présente invention se rapporte à une composition pharmaceutique utile pour la transfection d'un acide nucléique caractérisée en ce qu'elle contient outre ledit acide nucléique, au moins un agent de transfection et un composé intervenant au niveau de la condensation dudit acide nucléique, ledit composé dérivant en tout ou partie d'une histone, d'une nucléoline, d'une protamine et/ou de l'un de leurs dérivés.

15 De manière inattendue, la demanderesse a démontré que la présence d'un tel composé, au sein d'une composition transfectante à base d'un agent de transfection classique, permettait de diminuer considérablement la quantité en cet agent, avec les conséquences bénéfiques qui en découlent sur le plan toxicologique, sans porter un préjudice quelconque à l'activité transfectante de ladite composition. Au contraire,

20 celle-ci possède avantageusement un niveau de transfection supérieur.

Au sens de l'invention, un composé intervenant au niveau de la condensation de l'acide nucléique, couvre tout composé compactant, directement ou non, l'acide nucléique. Plus précisément, ce composé peut soit agir directement au niveau de l'acide nucléique à transfecter soit intervenir au niveau d'un composé annexe qui lui est

25 directement impliqué dans la condensation de cet acide nucléique. De préférence, il agit directement au niveau de l'acide nucléique.

Selon un mode de réalisation particulier de l'invention, le composé intervenant au niveau de la condensation de l'acides nucléique est constitué, en tout ou partie, de motifs peptidiques (KTPKKAKKP) SEQ ID N°1 et/ou (ATPAKKAA) SEQ ID N°2 ou

30 un de leurs dérivés, le nombre des motifs pouvant varier entre 2 et 10. Dans la structure du composé selon l'invention, ces motifs peuvent être répétés de manière

plan de la transfection mais on ne peut malheureusement exclure totalement à leur égard certains risques de pathogénicité, répllication et/ou immunogénicité, inhérents à leur nature virale.

5       La seconde stratégie consiste avantageusement à utiliser des agents non viraux capables de promouvoir le transfert et l'expression d'ADN dans des cellules eucaryotes.

L'objet de la présente invention s'inscrit plus particulièrement dans cette seconde stratégie.

10       Les vecteurs chimiques ou biochimiques représentent une alternative avantageuse aux virus naturels en particulier pour cette absence de réponse immunologique et/ou recombinaison virale. Ils ne possèdent pas de pouvoir pathogène, le risque de multiplication de l'ADN au sein de ces vecteurs est nul et il ne leur est attaché aucune limite théorique en ce qui concerne la taille de l'ADN à transférer.

15       Ces vecteurs synthétiques ont deux fonctions principales, condenser l'ADN à transférer et promouvoir sa fixation cellulaire ainsi que son passage à travers la membrane plasmique et, le cas échéant, les deux membranes nucléaires.

20       De part sa nature polyanionique, l'ADN n'a naturellement aucune affinité pour la membrane plasmique des cellules également polyanionique. Pour pallier à cet inconvénient, les vecteurs non viraux possèdent généralement tous des charges polycationiques.

25       Parmi les vecteurs synthétiques développés, les polymères cationiques de type polylysine et DEAE dextran ou encore les lipides cationiques ou lipofectants sont les plus avantageux. Ils possèdent la propriété de condenser l'ADN et de promouvoir son association avec la membrane cellulaire. Plus récemment, il a été développé le concept de la transfection ciblée, médiée par un récepteur. Cette technique met à profit le principe de condenser l'ADN, grâce au polymère cationique, tout en dirigeant la fixation du complexe à la membrane à l'aide d'un couplage chimique entre le polymère cationique et le ligand d'un récepteur membranaire, présent à la surface du type  
30       cellulaire que l'on veut greffer. Les ciblage du récepteur à la transferrine, à l'insuline ou du récepteur des asialoglycoprotéines des hépatocytes ont ainsi été décrits.

COMPOSITION CONTENANT DES ACIDES NUCLEIQUES.  
PREPARATION ET UTILISATION

La présente invention concerne le domaine de la thérapie génique et s'intéresse plus particulièrement au transfert in vitro, ex vivo et/ou in vivo du matériel génétique.

- 5 Elle propose notamment une nouvelle composition pharmaceutique utile pour transférer efficacement des cellules. Elle se rapporte également aux utilisations de cette composition.

- Des déficiences et/ou anomalies (mutation, expression aberrante, etc) chromosomiques sont à l'origine de nombreuses maladies, à caractère héréditaire ou  
10 non. Pendant longtemps, la médecine conventionnelle est demeurée impuissante à leur égard. Aujourd'hui, avec le développement de la thérapie génique, on espère pouvoir désormais corriger ou prévenir ce type d'aberration chromosomique. Cette nouvelle médication consiste à introduire une information génétique, dans la cellule ou l'organe affecté, en vu de corriger cette déficience ou anomalie ou encore, d'y exprimer une  
15 protéine d'intérêt thérapeutique.

L'obstacle majeur à la pénétration d'un acide nucléique dans une cellule ou un organe cible, repose sur la taille et la nature polyanionique de cet acide nucléique qui s'opposent à son passage à travers les membranes cellulaires.

- Pour lever cette difficulté, diverses techniques sont aujourd'hui proposées dont  
20 plus particulièrement la transfection d'ADN nu à travers la membrane plasmique in vivo (WO90/11092) et la transfection d'ADN via un vecteur de transfection.

- En ce qui concerne, la transfection d'ADN nu, son efficacité demeure encore très faible. Les acides nucléiques nus possèdent une demi vie plasmatique courte en raison de leur dégradation par les enzymes et de leur élimination par les voies  
25 urinaires.

Pour ce qui est de la seconde technique, elle propose principalement deux stratégies:

- La première met en oeuvre les vecteurs de transfection naturels que sont les virus. Il est ainsi proposé d'utiliser les adénovirus, les herpès virus, les rétrovirus et  
30 plus récemment les virus adéno associés. Ces vecteurs s'avèrent performants sur le



# **UNIQUEMENT A TITRE D'INFORMATION**

Codes utilisés pour identifier les Etats parties au PCT, sur les pages de couverture des brochures publiant des demandes internationales en vertu du PCT.

AT	Arménie	GB	Royaume-Uni	MW	Malawi
AT	Autriche	GE	Géorgie	MX	Mexique
AU	Australie	GN	Guinée	NE	Niger
BB	Barbade	GR	Grèce	NL	Pays-Bas
BE	Belgique	HU	Hongrie	NO	Norvège
BF	Burkina Faso	IE	Irlande	NZ	Nouvelle-Zélande
BG	Bulgarie	IT	Italie	PL	Pologne
BJ	Bénin	JP	Japon	PT	Portugal
BR	Brésil	KE	Kenya	RO	Roumanie
BY	Bélarus	KG	Kirghizistan	RU	Fédération de Russie
CA	Canada	KP	République populaire démocratique de Corée	SD	Soudan
CF	République centrafricaine	KR	République de Corée	SE	Suède
CG	Congo	KZ	Kazakhstan	SG	Singapour
CH	Suisse	LI	Liechtenstein	SI	Slovénie
CI	Côte d'Ivoire	LK	Sri Lanka	SK	Slovaquie
CM	Cameroun	LR	Libéria	SN	Sénégal
CN	Chine	LT	Lituanie	SZ	Swaziland
CS	Tchécoslovaquie	LU	Luxembourg	TD	Tchad
CZ	République tchèque	LV	Lettonie	TG	Togo
DE	Allemagne	MC	Monaco	TJ	Tadjikistan
DK	Danemark	MD	République de Moldova	TT	Trinité-et-Tobago
EE	Estonie	MG	Madagascar	UA	Ukraine
ES	Espagne	ML	Mali	UG	Ouganda
FI	Finlande	MN	Mongolie	US	Etats-Unis d'Amérique
FR	France	MR	Mauritanie	UZ	Ouzbékistan
GA	Gabon			VN	Viet Nam



## DEMANDE INTERNATIONALE PUBLIÉE EN VERTU DU TRAITE DE COOPERATION EN MATIERE DE BREVETS (PCT)

<b>(51) Classification internationale des brevets <sup>6</sup> :</b> C12N 15/87, A61K 48/00	<b>A1</b>	<b>(11) Numéro de publication internationale:</b> WO 96/25508 <b>(43) Date de publication internationale:</b> 22 août 1996 (22.08.96)
<b>(21) Numéro de la demande internationale:</b> PCT/FR96/00248 <b>(22) Date de dépôt international:</b> 15 février 1996 (15.02.96) <b>(30) Données relatives à la priorité:</b> 95/01865 17 février 1995 (17.02.95) FR <b>(71) Déposant (pour tous les Etats désignés sauf US):</b> RHONE-POULENC RORER S.A. [FR/FR]; 20, avenue Raymond-Aron, F-92160 Antony (FR). <b>(72) Inventeurs; et</b> <b>(75) Inventeurs/Déposants (US seulement):</b> BYK, Gerardo [IL/FR]; 3, impasse Eugène-Delacroix, F-94000 Créteil (FR). SCHERMAN, Daniel [FR/FR]; 50, rue du Disque, F-75645 Paris Cédex 13 (FR). SCHWARTZ, Bertrand [FR/FR]; 13-15, rue Paul-Bert, F-94700 Maisons-Alfort (FR). <b>(74) Mandataire:</b> LE COUPANEC, Pascale; Rhône-Poulenc Rorer S.A., Direction Brevets, 20, avenue Raymond-Aron, F-92165 Antony Cédex (FR).		<b>(81) Etats désignés:</b> AL, AM, AU, BB, BG, BR, CA, CN, CZ, EE, FI, GE, HU, IS, JP, KP, KR, LK, LR, LT, LV, MD, MG, MK, MN, MX, NO, NZ, PL, RO, SG, SI, SK, TR, TT, UA, US, UZ, VN, brevet ARIPO (KE, LS, MW, SD, SZ, UG), brevet eurasien (AZ, BY, KG, KZ, RU, TJ, TM), brevet européen (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), brevet OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).  <b>Publiée</b> <i>Avec rapport de recherche internationale. Avant l'expiration du délai prévu pour la modification des revendications, sera republiée si de telles modifications sont reçues.</i>

**(54) Title:** NUCLEIC ACID-CONTAINING COMPOSITION, PREPARATION AND USE THEREOF**(54) Titre:** COMPOSITIONS CONTENANT DES ACIDES NUCLEIQUES, PREPARATION ET UTILISATION**(57) Abstract**

Pharmaceutical composition useful for transfecting a nucleic acid and characterised in that it contains, in addition to said nucleic acid, at least one transfecting agent and a compound causing the condensation of said nucleic acid, wherein said compound is totally or partly derived from a histone, a nucleoline, a protamine and/or a derivative thereof. The use of said composition for transferring nucleic acids in vitro, ex vivo and/or in vivo is also described.

**(57) Abrégé**

La présente invention concerne une composition pharmaceutique utile pour la transfection d'un acide nucléique caractérisée en ce qu'elle contient outre ledit acide nucléique, au moins un agent de transfection et un composé intervenant au niveau de la condensation dudit acide nucléique, ledit composé dérivant en tout ou partie d'une histone, d'une nucléoline, d'une protamine et/ou de l'un de leurs dérivés. Elle se rapporte en outre à l'utilisation de ladite composition pour le transfert in vitro, ex vivo et/ou in vivo d'acides nucléiques.

most important and life threatening pathology occurs in the lung. Gene therapy has been proposed as a means of developing effective therapy to combat the pathology of CF. However, there are a plethora of problems associated with this approach, not the least of which is the lack of a suitable vehicle for delivery of the CFTR gene to humans.

Initial reports of gene therapy as a means of treating CF have focussed on airway epithelial cells as targets for the CFTR gene. Viral vectors have been used as vehicles for delivery of the CFTR gene to these cells in humans. However, the vectors themselves have proved to be sufficiently immunogenic so as to diminish any positive effect of the successful delivery of the CFTR gene to the affected cells of the individual (Wilson, 1995, *J. Clin. Invest.* **96**:2547-2554; Crystal *et al.*, 1995, *Science* **270**:404-410).

Other vehicles which have been used as gene delivery vehicles include cationic lipids for transfer of genes to airway epithelial cells (Fasbender *et al.*, 1995, *Am. J. Physiol.* **269**:L45-L51). In addition, polylysines (poly-L-lysine) complexed with various glycoproteins, including transferrin targeted to the transferrin receptor, have been examined (Curiel *et al.*, 1991, *Proc. Natl. Acad. Sci. USA* **88**:8850-8854). Also reported, is the use of asialoglycoproteins targeted to hepatic cells through the asialoglycoprotein receptor (Wilson *et al.*, 1992, *J. Biol. Chem.* **267**:963-967), and the use of Tn antigen for gene transfer (Thurnher *et al.*, 1994, *Glycobiology* **4**:429-435). The above-referenced studies have largely been performed in cells other than airway epithelial cells. Moreover, complexes having glycoprotein as a component thereof are potentially immunogenic and therefore may not be of immediate value in human gene therapy.

Asthma is a disease of the industrialized 20th century, being described for the first time in the mid-1800's. Exposure to otherwise harmless pollens and other allergens may set off a life threatening asthma attack in susceptible individuals, wherein constriction of the bronchioles renders a patient virtually unable to breathe. Asthma attacks are triggered by exposure to allergens which cause activated T lymphocytes of the  $T_H2$  subset to secrete cytokines, primarily interleukin 4 (IL-4) and interleukin 5 (IL-5) setting off a cascade of events

## NON-VIRAL VEHICLES FOR USE IN GENE TRANSFER

## FIELD OF THE INVENTION

5           The field of the invention is delivery of genes to cells *in vitro* and *in vivo*, in particular, non-viral delivery of genes to cells.

## GOVERNMENT SUPPORT

          Portions of this invention were supported by a grant from the U.S.  
10   Government (NIH Grant No. RO1 16859) and the U.S. Government may therefore have certain rights in the invention.

## BACKGROUND OF THE INVENTION

          Diseases of the respiratory tract are among the most common  
15   diseases in humans and range in severity from being merely mild and annoying to life threatening. Examples of severe to life threatening respiratory diseases in humans include cystic fibrosis, asthma, emphysema, idiopathic pulmonary fibrosis and congenital deficiency of surfactant protein. Each of these diseases is suitable for gene therapy as a means of treatment provided that a gene delivery vehicle is  
20   available which delivers the appropriate gene in an effective and non-toxic manner. Gene therapy approaches which have been used or are contemplated for treatment of these respiratory diseases are reviewed in Canonico (1997, Gene Therapy for Chronic Inflammatory Diseases of the Lungs in *Gene Therapy for Diseases of the Lung*, K.L. Brigham, ed, Marcel Dekker, New York, pp.285-307).

25           Cystic fibrosis (CF) is the most common lethal genetic disease in Caucasians and, although the average life expectancy has increased to approximately 30 years in the past decade, there remains no effective cure for CF (Scanlin *et al.*, 1988, A.P. Fishman, ed. (McGraw-Hill, New York) pp. 1273-1294; Welsh *et al.*, 1995, Scriber *et al.*, eds. (McGraw-Hill, New York) pp. 3799-3876).

30           Patients having CF encode a mutated cystic fibrosis transmembrane conductance regulator (CFTR) gene. Although CF is a multisystem disease, the

**FOR THE PURPOSES OF INFORMATION ONLY**

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	ES	Spain	LS	Lesotho	SI	Slovenia
AM	Armenia	FI	Finland	LT	Lithuania	SK	Slovakia
AT	Austria	FR	France	LU	Luxembourg	SN	Senegal
AU	Australia	GA	Gabon	LV	Latvia	SZ	Swaziland
AZ	Azerbaijan	GB	United Kingdom	MC	Monaco	TD	Chad
BA	Bosnia and Herzegovina	GE	Georgia	MD	Republic of Moldova	TG	Togo
BB	Barbados	GH	Ghana	MG	Madagascar	TJ	Tajikistan
BE	Belgium	GN	Guinea	MK	The former Yugoslav Republic of Macedonia	TM	Turkmenistan
BF	Burkina Faso	GR	Greece			TR	Turkey
BG	Bulgaria	HU	Hungary	ML	Mali	TT	Trinidad and Tobago
BJ	Benin	IE	Ireland	MN	Mongolia	UA	Ukraine
BR	Brazil	IL	Israel	MR	Mauritania	UG	Uganda
BY	Belarus	IS	Iceland	MW	Malawi	US	United States of America
CA	Canada	IT	Italy	MX	Mexico	UZ	Uzbekistan
CF	Central African Republic	JP	Japan	NE	Niger	VN	Viet Nam
CG	Congo	KE	Kenya	NL	Netherlands	YU	Yugoslavia
CH	Switzerland	KG	Kyrgyzstan	NO	Norway	ZW	Zimbabwe
CI	Côte d'Ivoire	KP	Democratic People's Republic of Korea	NZ	New Zealand		
CM	Cameroon			PL	Poland		
CN	China	KR	Republic of Korea	PT	Portugal		
CU	Cuba	KZ	Kazakhstan	RO	Romania		
CZ	Czech Republic	LC	Saint Lucia	RU	Russian Federation		
DE	Germany	LI	Liechtenstein	SD	Sudan		
DK	Denmark	LK	Sri Lanka	SE	Sweden		
EE	Estonia	LR	Liberia	SG	Singapore		



## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

<b>(51) International Patent Classification <sup>6</sup> :</b> <b>C12P 21/06, C12N 5/00, 15/00, C07H</b> <b>21/02, 21/04, A01N 43/04</b>		<b>A1</b>	<b>(11) International Publication Number:</b> <b>WO 98/06869</b>
			<b>(43) International Publication Date:</b> 19 February 1998 (19.02.98)
<b>(21) International Application Number:</b> PCT/US97/14280		<b>(81) Designated States:</b> AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, HU, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, UZ, VN, YU, ZW, ARIPO patent (GH, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).	
<b>(22) International Filing Date:</b> 13 August 1997 (13.08.97)		<b>Published</b> <i>With international search report.</i> <i>Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>	
<b>(30) Priority Data:</b> 60/023,941 14 August 1996 (14.08.96) US 08/907,673 8 August 1997 (08.08.97) US			
<b>(71) Applicant:</b> THE CHILDREN'S HOSPITAL OF PHILADELPHIA [US/US]; 34th Street & Civic Center Boulevard, Philadelphia, PA 19104-4318 (US).			
<b>(72) Inventors:</b> GLICK, Mary, Catherine; 2028 Delancey Place, Philadelphia, PA 19104 (US). SCANLIN, Thomas, F.; 4250 Regent Square, Philadelphia, PA 19104 (US). KOLLEN, Wouter, J., W.; 415 South Van Pelt Street B-2, Philadelphia, PA 19146 (US).			
<b>(74) Agents:</b> LEARY, Kathryn, Doyle et al.; Panitch Schwarze Jacobs & Nadel, P.C., 36th floor, 1601 Market Street, Philadelphia, PA 19103-2398 (US).			
<b>(54) Title:</b> NON-VIRAL VEHICLES FOR USE IN GENE TRANSFER			
<b>(57) Abstract</b> <p>The invention relates to compositions and methods for transfection of cells, particularly airway epithelial cells, with DNA complexed to polylysine substituted with glycosyl residues. The invention also relates to methods of treating humans having respiratory disease comprising administering to a human the composition of the invention.</p>			



INTERNATIONAL SEARCH REPORT

International application No. -

PCT/US97/14280

A. CLASSIFICATION OF SUBJECT MATTER:

IPC (6):

C12P 21/06; C12N 5/00, 15/00; C07H 21/02, 21/04; A01N 43/04

A. CLASSIFICATION OF SUBJECT MATTER:

US CL :

435/6, 69.1, 172.1, 172.3; 514/2, 44; 536/23.1, 23.2, 23.4, 320.1, 325



## INTERNATIONAL SEARCH REPORT

International application No. -

PCT/US97/14280

## C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	ERBACHER, P. et al. Glycosylated Polylysine/DNA Complexes: Gene Transfer Efficiency in Relation with the Size and the Sugar Substitution Level of Glycosylated Polylysines and with the Plasmid Size. Bioconjugate Chemistry. 1995, Vol. 6, No. 4, pages 401-410, see entire document.	1-77

## INTERNATIONAL SEARCH REPORT

International application No. -

PCT/US97/14280

## A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) : Please See Extra Sheet.

US CL : Please See Extra Sheet.

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/6, 69.1, 172.1, 172.3; 514/2, 44; 536/23.1, 23.2, 23.4, 320.1, 325

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched  
NONEElectronic data base consulted during the international search (name of data base and, where practicable, search terms used)  
APS, MEDLINE, BIOSIS, SCISEARCH, EMBASE, CAPLUS

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	ERBACHER, P. et al. Putative Roles of Chloroquine in Gene Transfer into Human Hepatoma Cell Line by DNA/Lactosylated Polylysine Complexes. Experimental Cell Research. 1996, Vol. 225, pages 186-194, see entire document.	1-77
Y	ERBACHER, P. et al. Gene Transfer by DNA/Glycosylated Polylysine Complexes into Human Blood Monocyte-Derived Macrophages. Human Gene Therapy. 10 April 1996, Vol. 7, pages 721-729, see entire document.	1-77
Y	MIDOUX, P. et al. Specific Gene Transfer Mediated by Lactosylated Poly-L-Lysine into Hepatoma Cells. Nucleic Acids Research. 1993, Vol. 21, No. 4, pages 871-878, see entire document.	1-77



Further documents are listed in the continuation of Box C.



See patent family annex.

* Special categories of cited documents	*T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
*A* document defining the general state of the art which is not considered to be of particular relevance	*X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
*B* earlier document published on or after the international filing date	*Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
*L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	*A* document member of the same patent family
*O* document referring to an oral disclosure, use, exhibition or other means	
*P* document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search

29 SEPTEMBER 1997

Date of mailing of the international search report

14 JAN 1998

Name and mailing address of the ISA/US  
Commissioner of Patents and Trademarks  
Box PCT  
Washington, D.C. 20231

Facsimile No. (703) 305-3230

Authorized officer

SEAN MCGARRY

Telephone No. (703) 308-0196

18/18

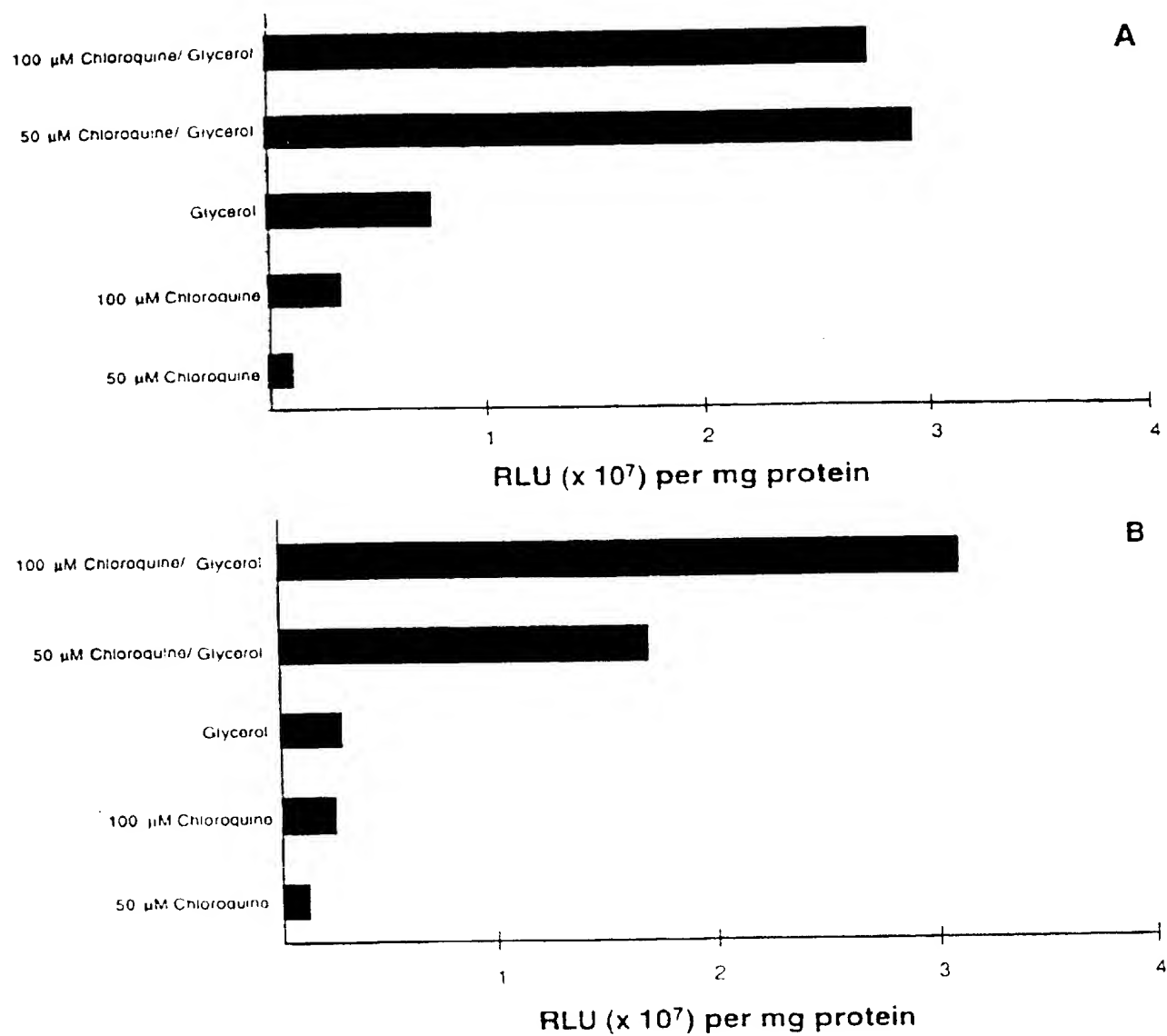


FIGURE 18

A



B

FIGURE 17

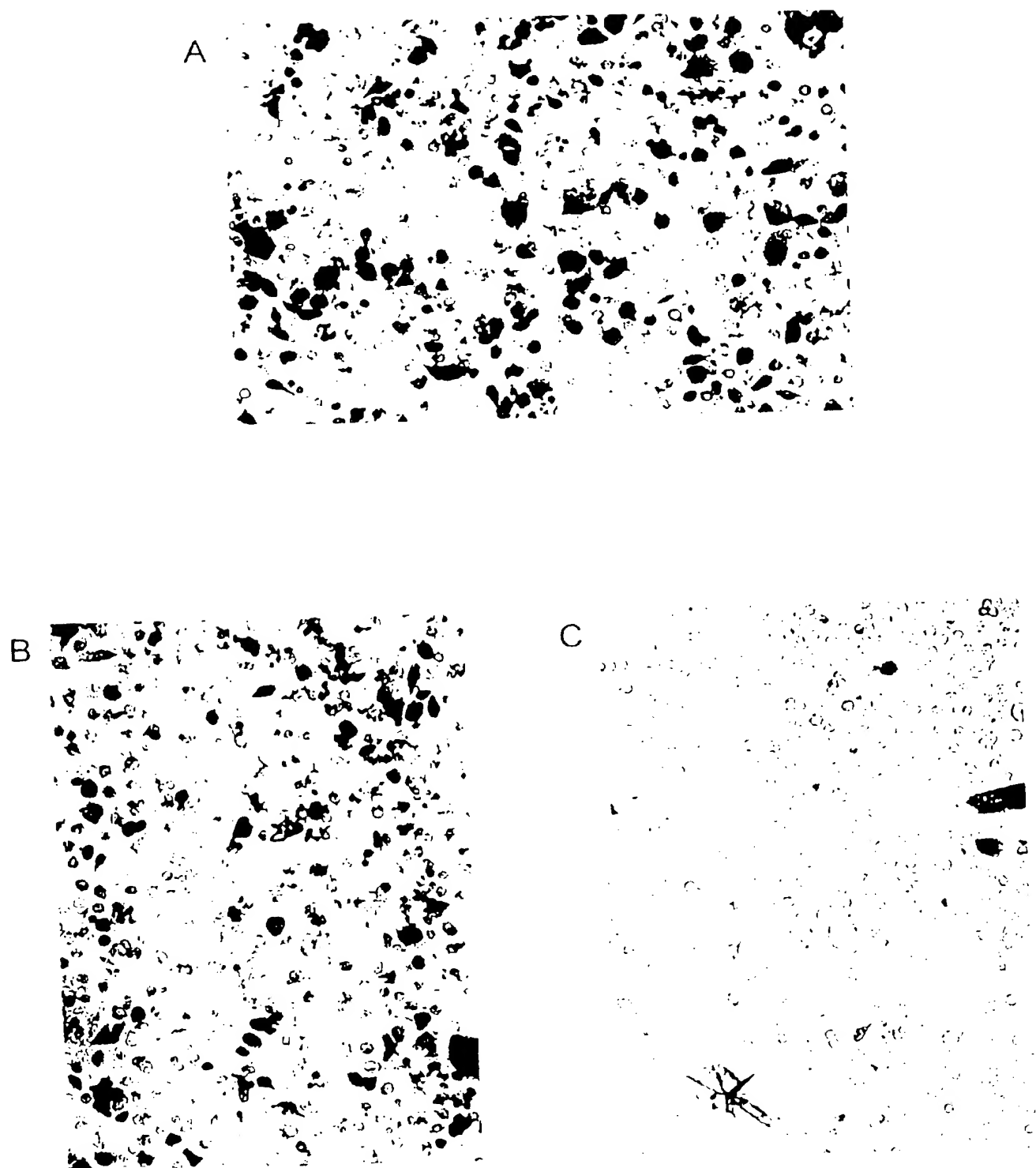


FIGURE 16

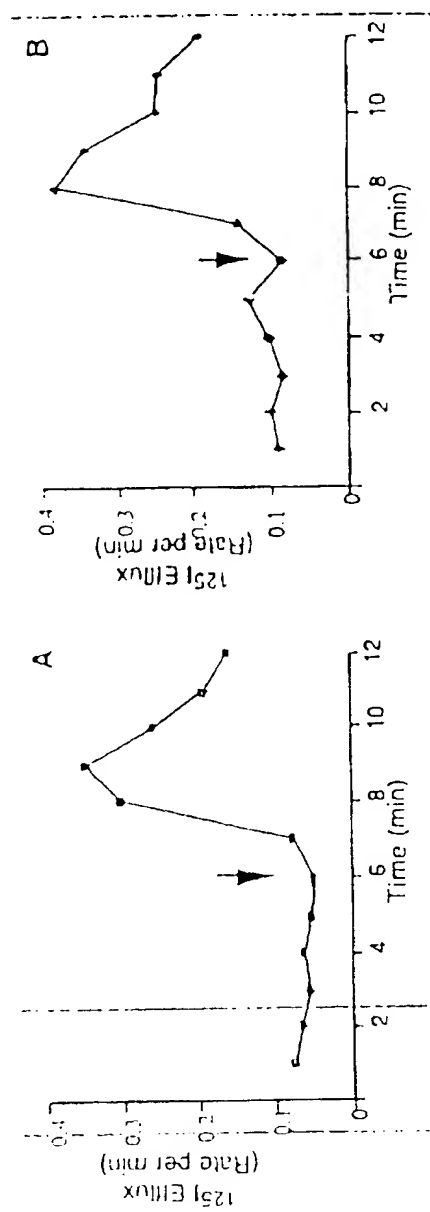


FIGURE 15

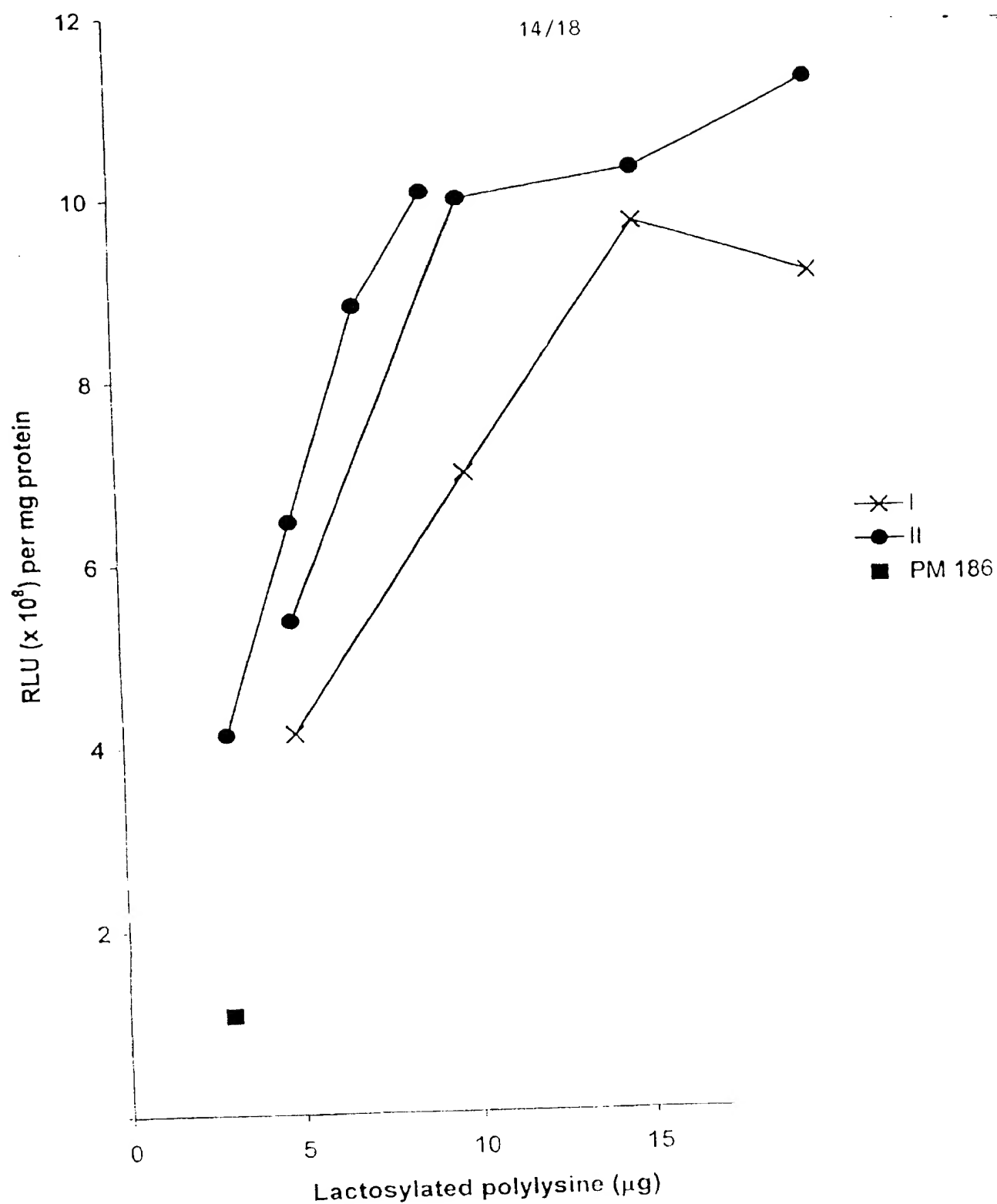


FIGURE 14

13/18

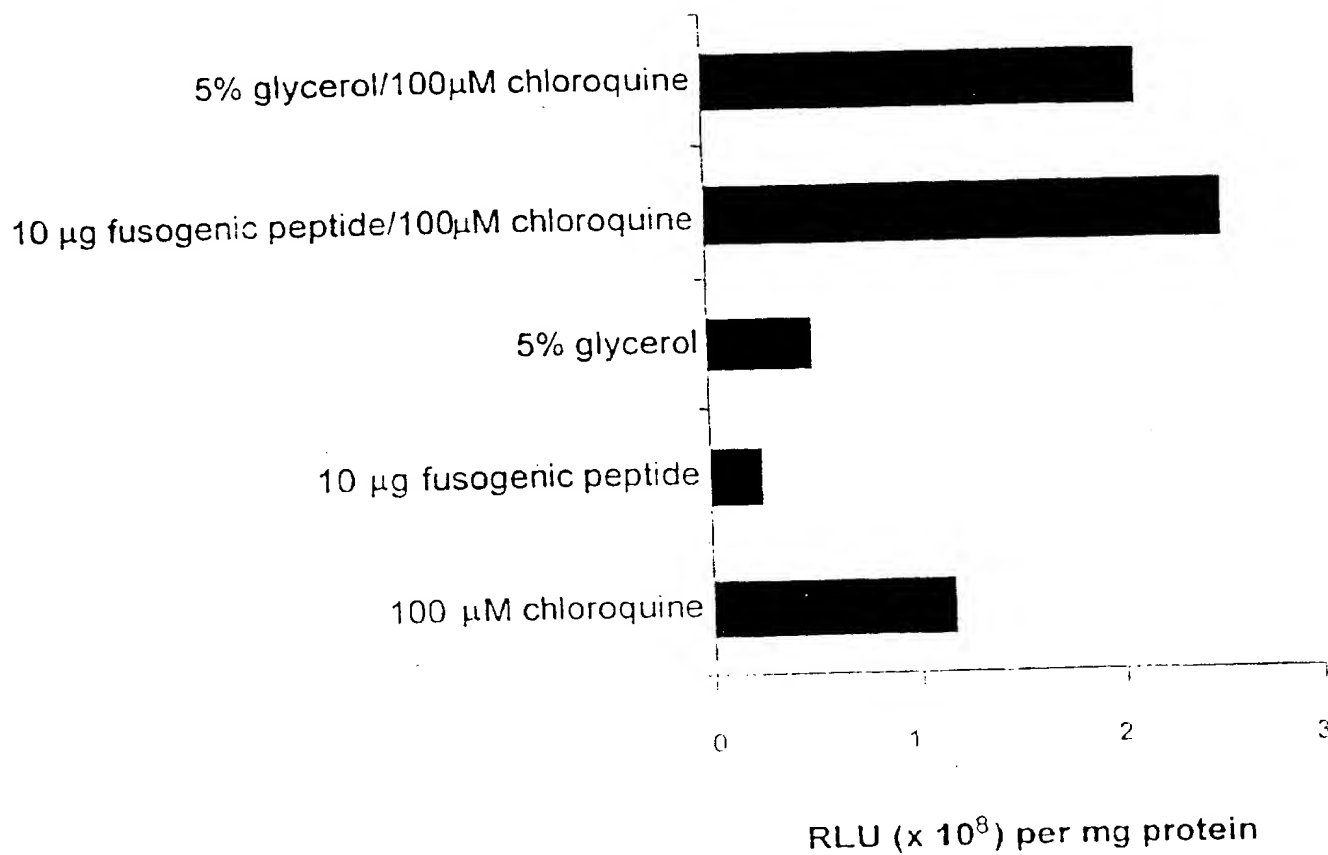


FIGURE 13



12/18

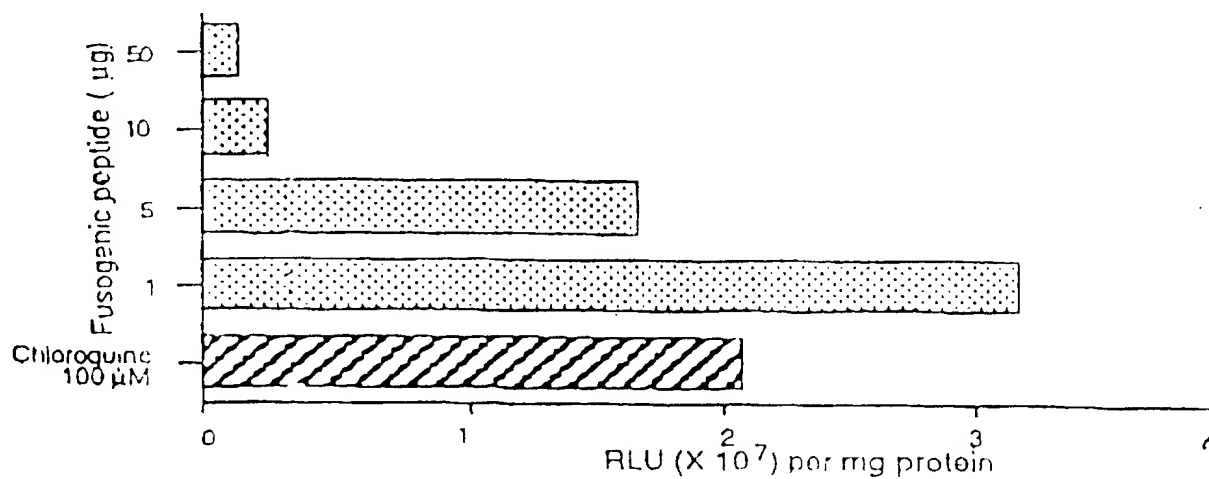


FIGURE 12

11/18

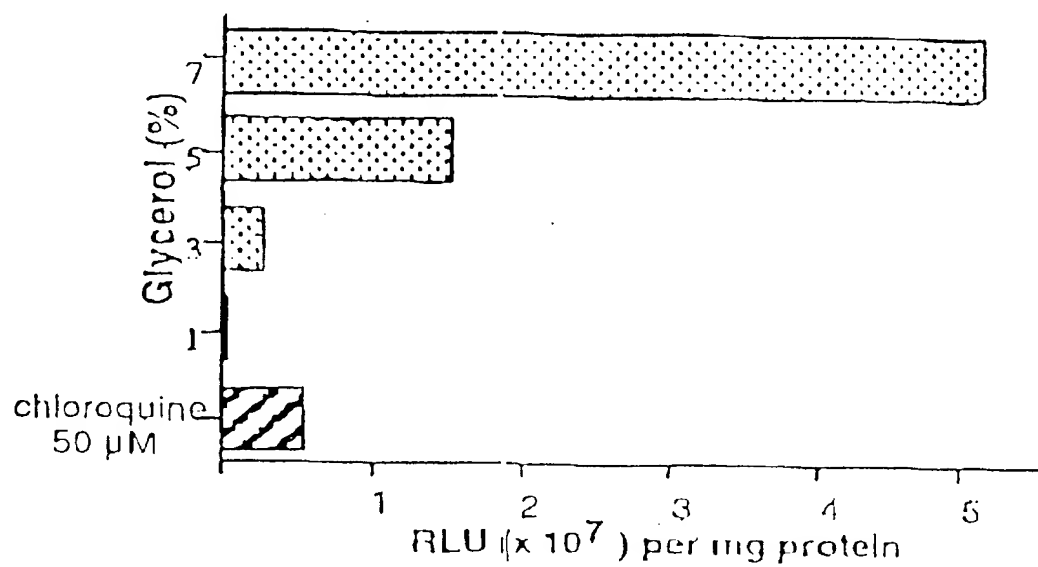


FIGURE 11

10/18

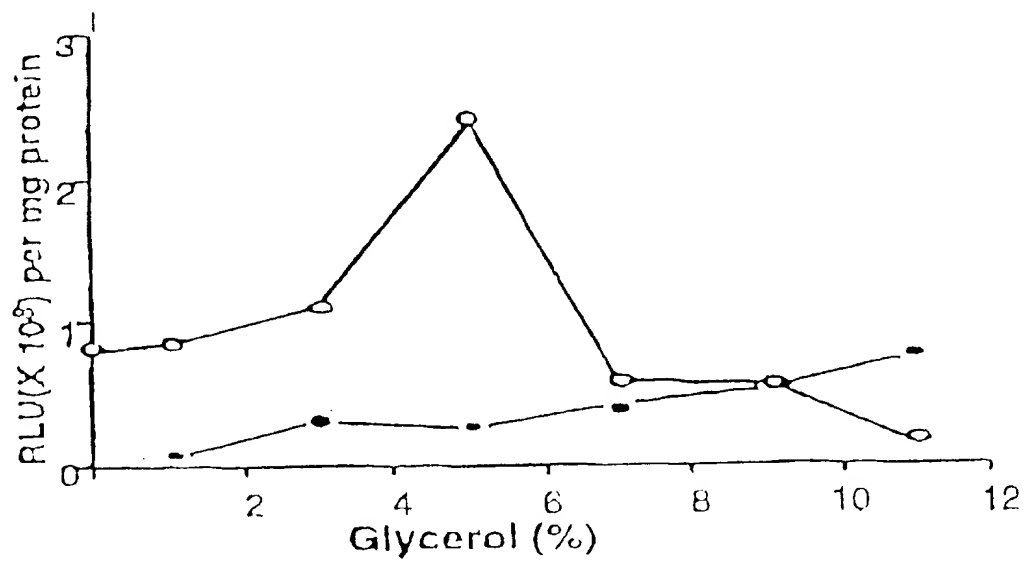


FIGURE 10

9/18

FIGURE 9A

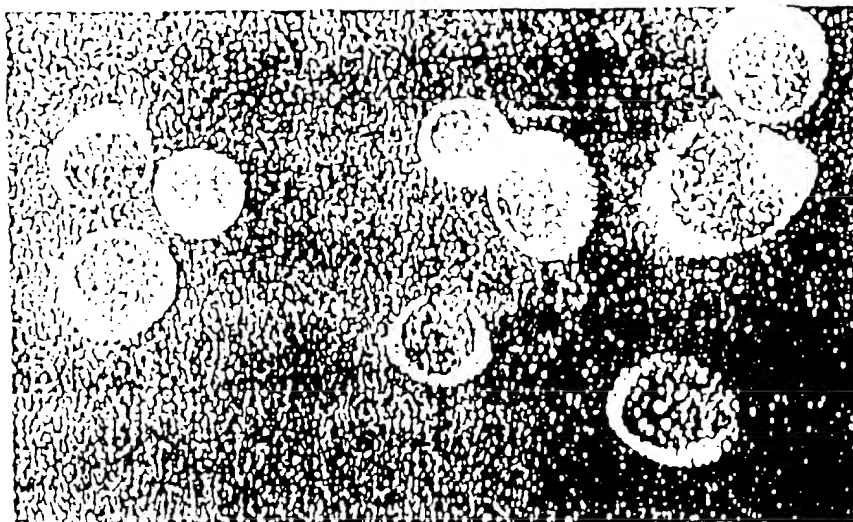
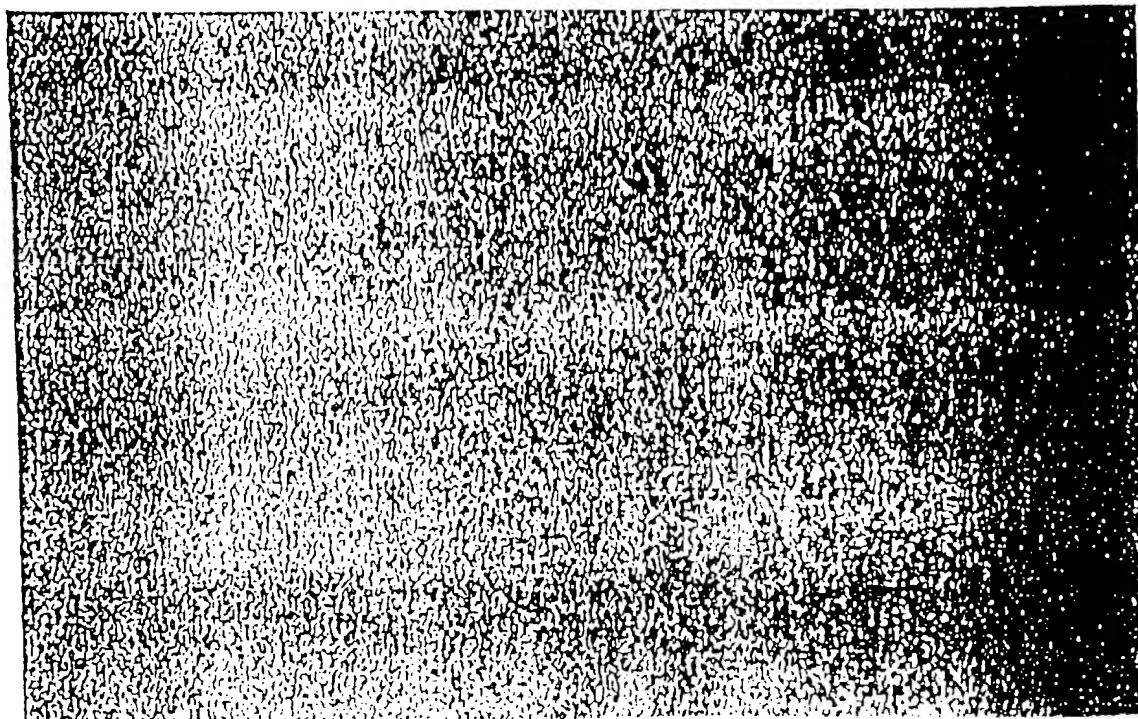


FIGURE 9B



8/18

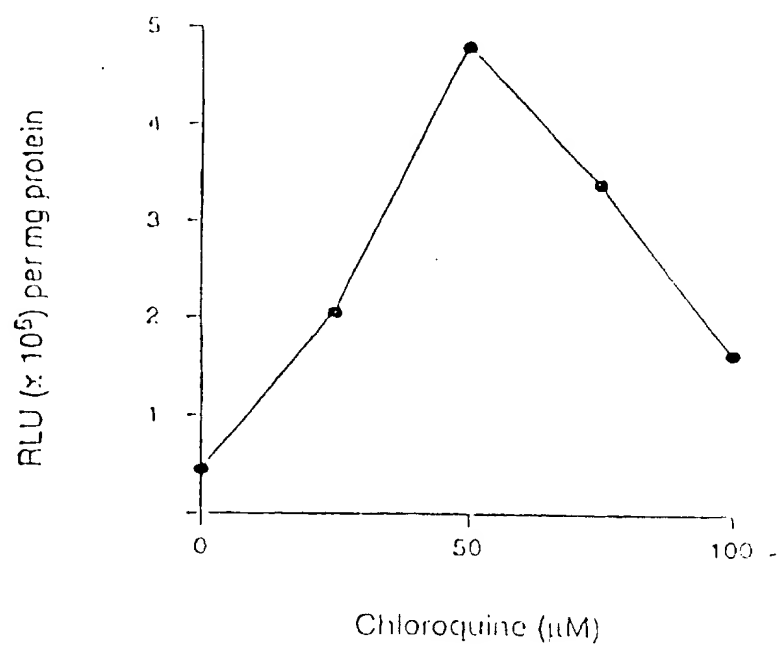


FIGURE 8

FIGURE 7A

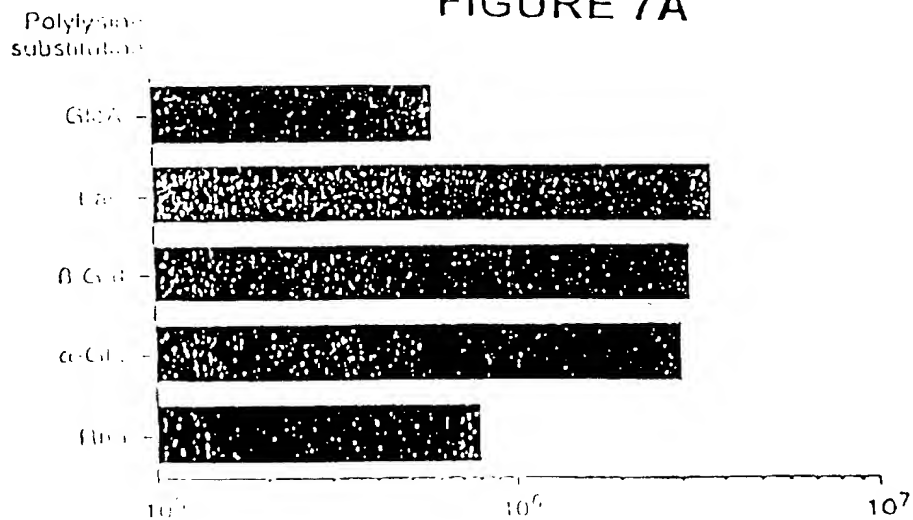
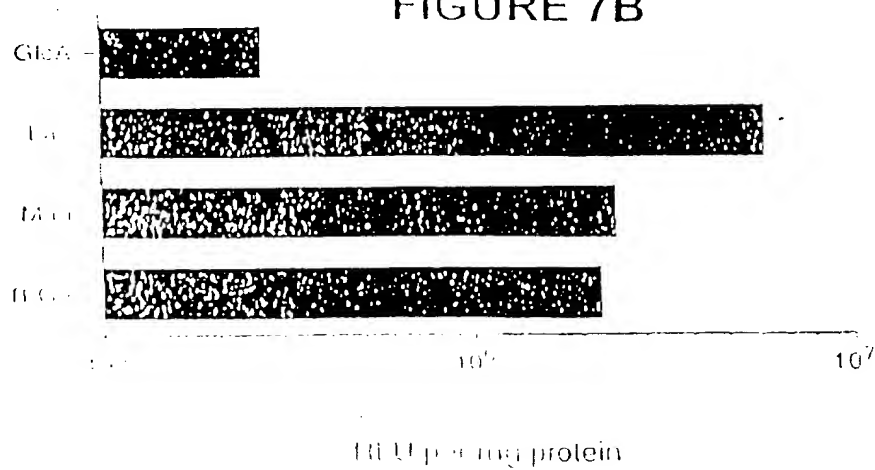


FIGURE 7B



6/18

FIGURE 6A

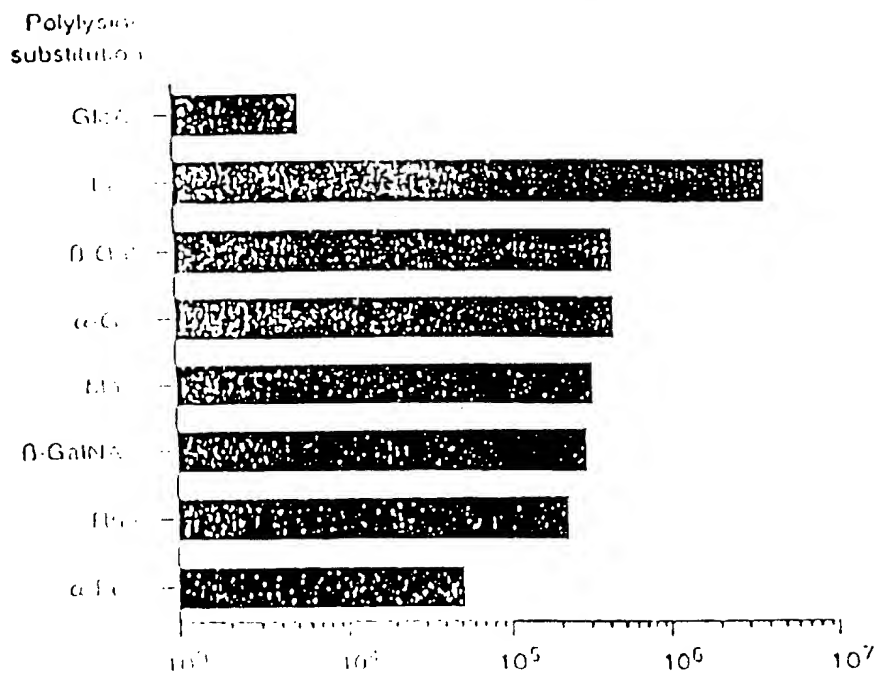
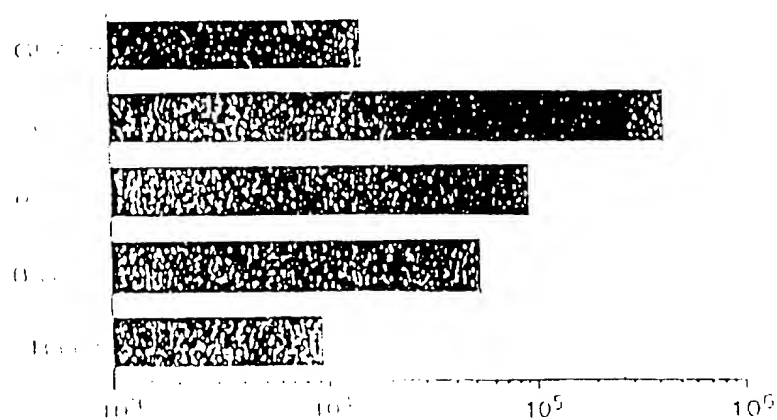


FIGURE 6B



RFU per mg protein

5/18

FIGURE 5A

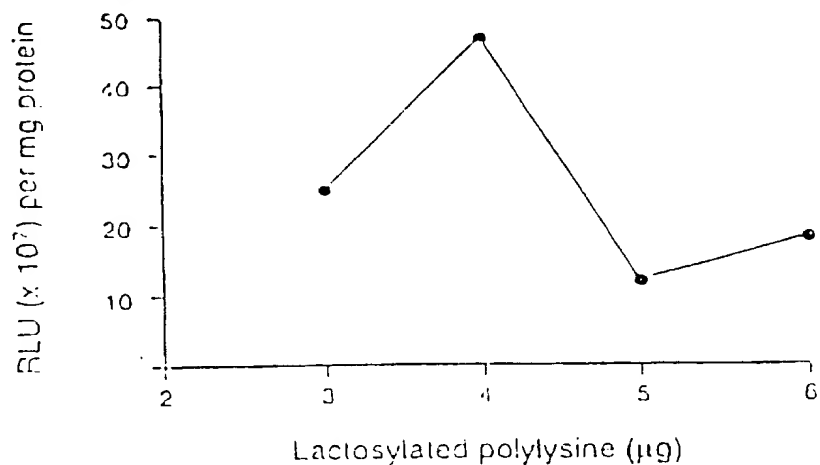


FIGURE 5B

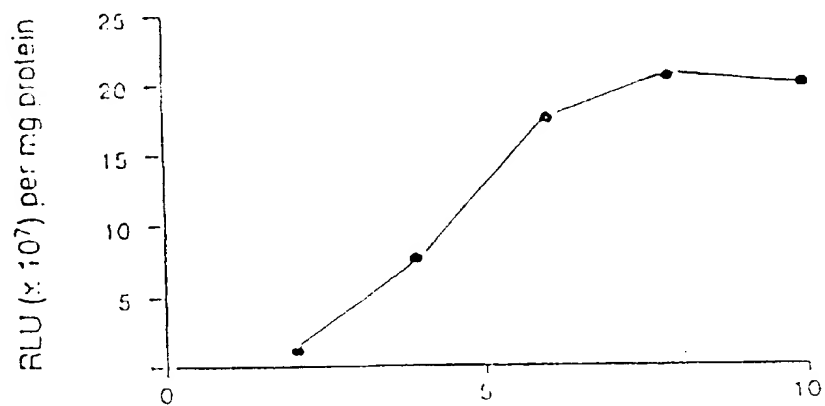
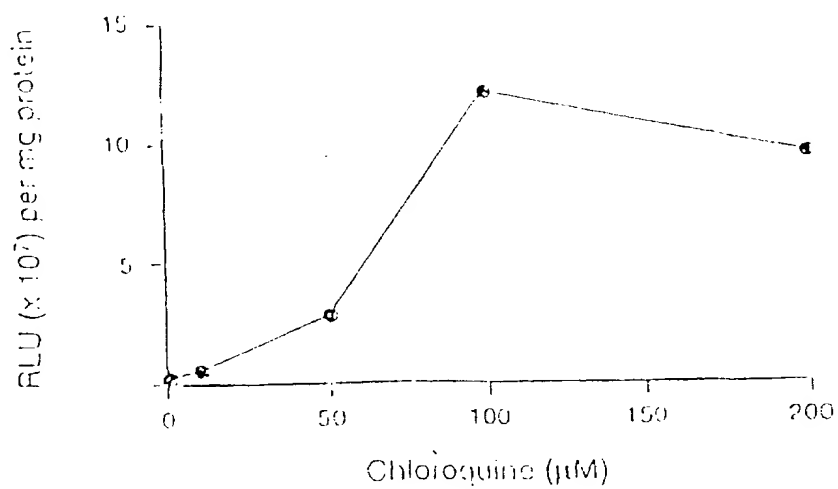


FIGURE 5C





4/18

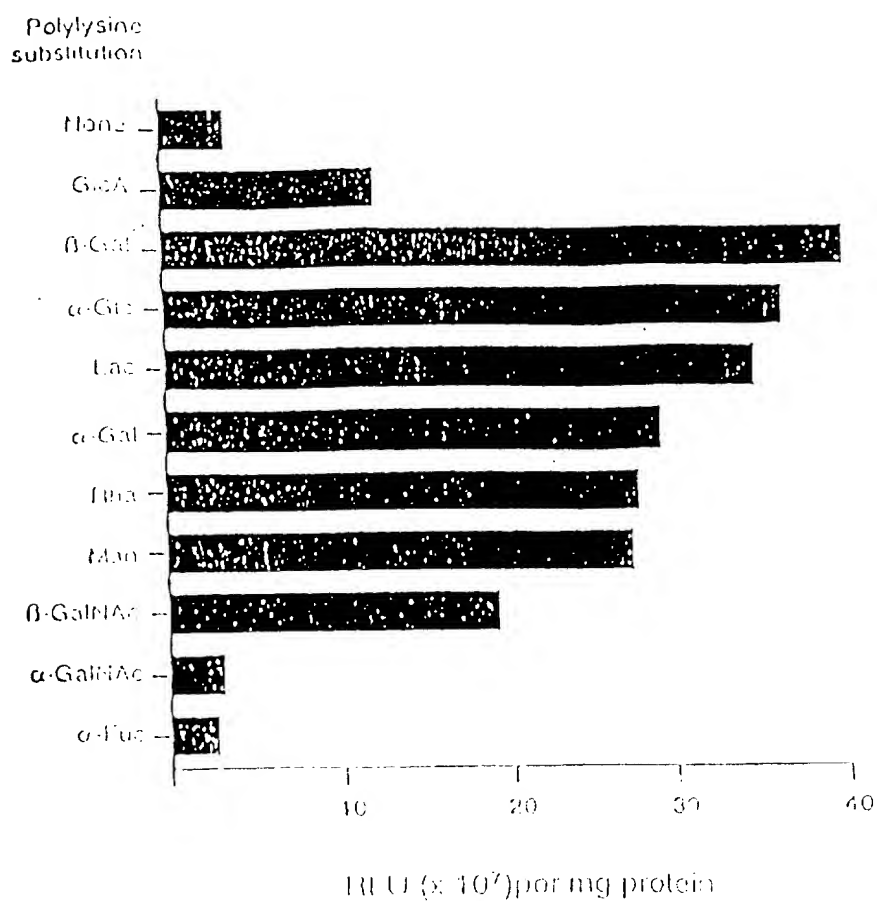


FIGURE 4

3/18

FIGURE 3A

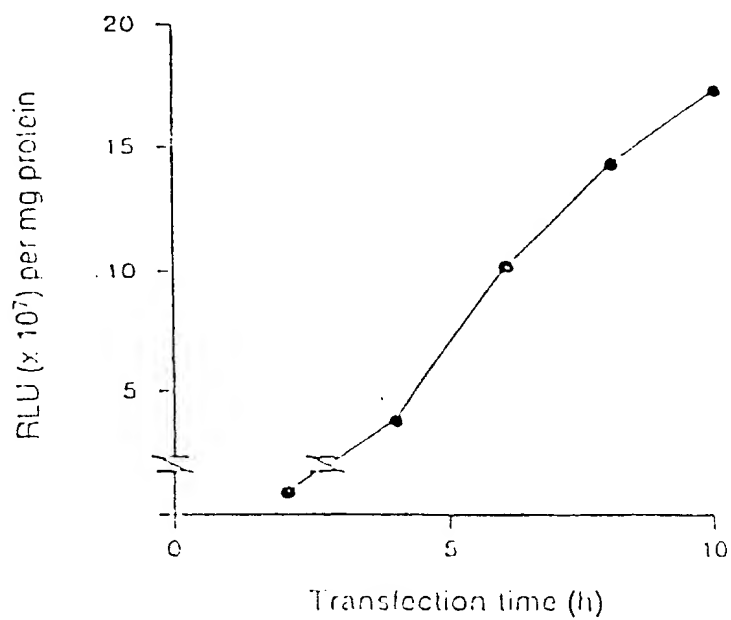
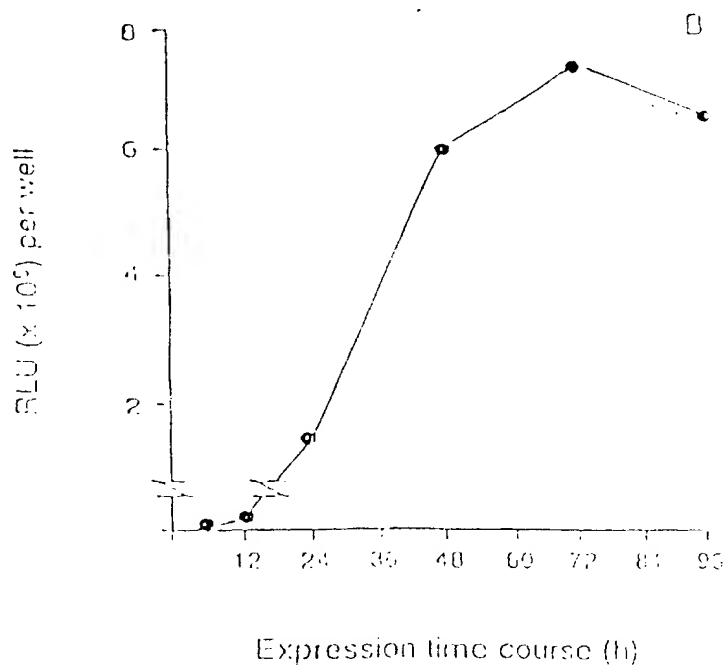


FIGURE 3B



2/18

FIGURE 2A

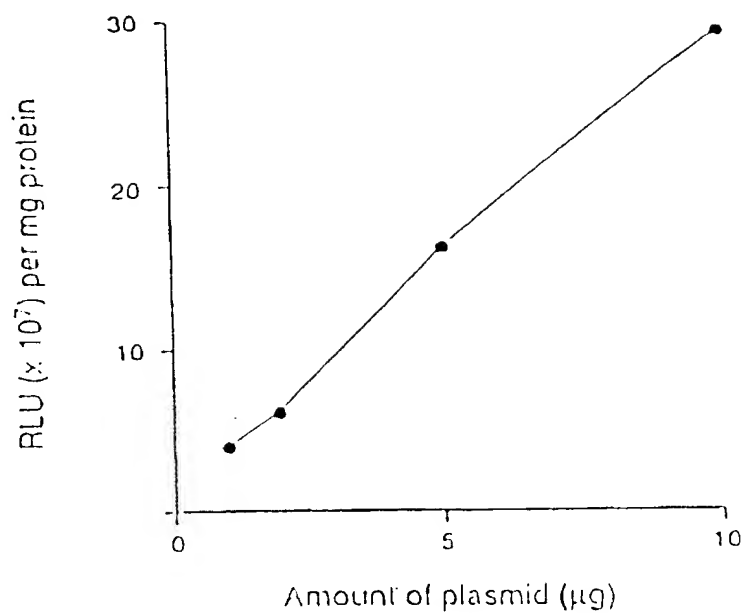
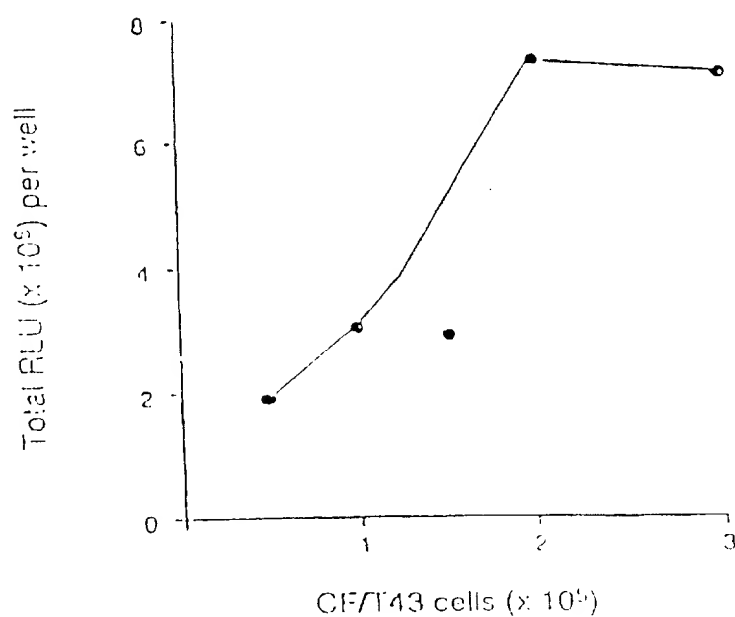


FIGURE 2B



1/18

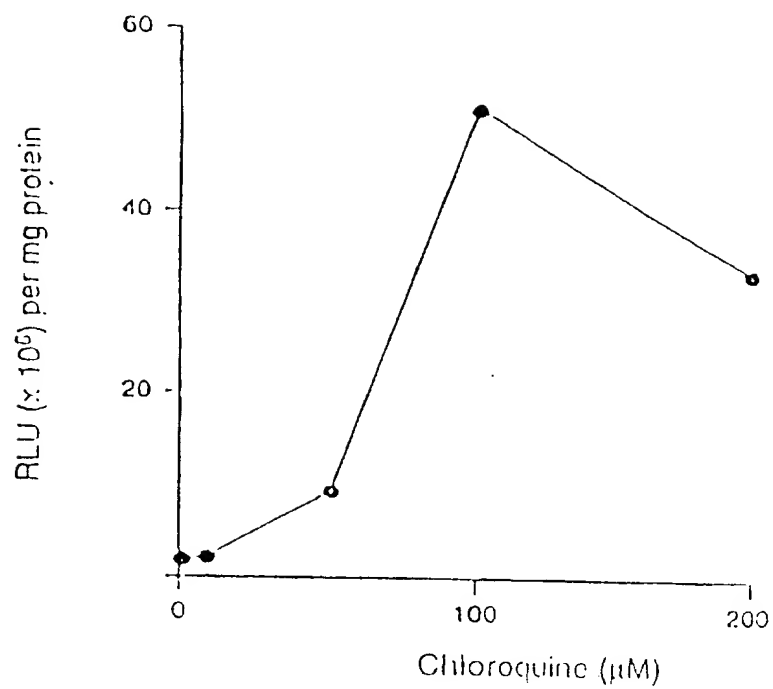


FIGURE 1

72. An airway epithelial cell transfected with a complex comprising an isolated nucleic acid and a glycosylated polylysine and at least one of chloroquine, glycerol and a fusogenic peptide.

5                   73. The airway epithelial cell of claim 72, wherein said isolated nucleic acid is DNA encoding CFTR and said glycosylated polylysine is lactosylated polylysine.

10                   74. A composition for transfection of airway epithelial cells comprising a complex comprising an isolated nucleic acid and a lactosylated polylysine, wherein said isolated nucleic acid is DNA selected from the group consisting of DNA encoding an asthma gene, DNA encoding  $\alpha 1$ AT, a gene affecting idiopathic pulmonary fibrosis, DNA encoding SP-B and DNA encoding SP-C.

15

75. The composition of claim 74, further comprising at least one of chloroquine, glycerol and a fusogenic peptide.

20                   76. The composition of claim 74, wherein said airway epithelial cells are transfected *in vitro*.

77. The composition of claim 74, wherein said airway epithelial cells are transfected *in vivo*.

25

63. An *in vitro* cell transfection kit comprising a selection of glycosylated polylysines and instructions for using said kit.

64. The kit of claim 63, further comprising a reporter DNA.

5

65. The kit of claim 63, further comprising at least one of chloroquine, glycerol and a fusogenic peptide.

10

66. The kit of claim 63, said glycosylated polylysine having a sugar component selected from the group consisting of lactose,  $\alpha$ -glucose,  $\beta$ -galactose, mannose, mannose-6-phosphate, fucose and N-acetylglucosamine.

15

67. The kit of claim 64, wherein said reporter DNA is selected from the group consisting of a chloramphenicol acetyl transferase gene, a luciferase gene, a green fluorescent protein gene, and a  $\beta$ -galactosidase gene.

20

68. A nebulizer having a composition comprising a complex comprising an isolated nucleic acid and a glycosylated polylysine placed therein.

69. The nebulizer of claim 68, wherein said isolated nucleic acid is DNA encoding CFTR and said glycosylated polylysine is lactosylated polylysine.

25

70. A bronchoscope having a composition comprising a complex comprising an isolated nucleic acid and a glycosylated polylysine placed therein.

71. The bronchoscope of claim 70, wherein said isolated nucleic acid is DNA encoding CFTR and said glycosylated polylysine is lactosylated polylysine.

56. The method of claim 51, said glycosylated polylysine having a sugar component selected from the group consisting of lactose,  $\alpha$ -glucose,  $\beta$ -galactose, mannose, mannose-6-phosphate, fucose and N-acetylglucosamine.

5 57. The method of claim 56, wherein said glycosylated polylysine comprises lactosylated polylysine.

58. The method of claim 57, wherein about 10% to about 60% of the amino groups of said polylysine have a lactose molecule substituted thereon.

10

59. The method of claim 57, wherein the weight to weight ratio of lactosylated polylysine to DNA in said complex is about one to one to about fifteen to one.

15

60. The method of claim 59, wherein the weight to weight ratio of lactosylated polylysine to DNA in said complex is about three to one to about nine to one.

20

61. A method of identifying a test compound capable of modulating the activity of CFTR comprising

transfecting airway epithelial cells in the presence or absence of said test compound with a complex comprising an isolated nucleic acid encoding CFTR, or a biologically active fragment thereof, and a glycosylated polylysine, and

25

measuring the activity of CFTR in said cells, wherein a higher or a lower level of CFTR activity in the presence of said test compound compared with CFTR activity in cells in the absence of said test compound is an indication that said test compound is capable of modulating the activity of CFTR.

30

62. A compound identified according to the method of claim 61.

48. The lactosylated polylysine nucleic acid complex of claim 47, wherein the weight to weight ratio of lactosylated polylysine to said isolated nucleic acid in said complex is about nine to one.

5 49. A kit comprising an isolated nucleic acid encoding CFTR, or a biologically active fragment thereof, a glycosylated polylysine and instructions for using said kit for transfection of airway epithelial cells.

10 50. A kit comprising an isolated nucleic acid encoding CFTR, or a biologically active fragment thereof, a glycosylated polylysine and instructions for using said kit for treatment of cystic fibrosis in a human patient.

15 51. A method of treating a human patient having cystic fibrosis, said method comprising administering to said human a pharmaceutical composition comprising a complex comprising an isolated nucleic acid encoding CFTR, or a biologically active fragment thereof, and a glycosylated polylysine.

20 52. The method of claim 51, said composition further comprising at least one of chloroquine, glycerol and a fusogenic peptide.

53. The method of claim 51, wherein said pharmaceutical composition is administered to said human by a means selected from the group consisting of aerosol nebulizer, bronchoscopy and injection *in utero*.

25 54. The method of claim 51, wherein said isolated nucleic acid comprises DNA.

30 55. The method of claim 51, wherein said isolated nucleic acid comprises cDNA.



41. The pharmaceutical composition of claim 37, further comprising at least one of chloroquine, glycerol and a fusogenic peptide.

5 42. The pharmaceutical composition of claim 37, said glycosylated polylysine having a sugar component selected from the group consisting of lactose,  $\alpha$ -glucose,  $\beta$ -galactose, mannose, mannose-6-phosphate, fucose and N-acetylglucosamine.

10 43. The pharmaceutical composition of claim 42, wherein said glycosylated polylysine comprises lactosylated polylysine.

15 44. The pharmaceutical composition of claim 43, wherein about 10% to about 60% of the amino groups of said polylysine have a lactose molecule substituted thereon.

45. The pharmaceutical composition of claim 44, wherein about 12% to about 40% of the amino groups of said polylysine have a lactose molecule substituted thereon.

20 46. The pharmaceutical composition of claim 43, wherein the weight to weight ratio of lactosylated polylysine to DNA in said complex is about one to one to about fifteen to one.

25 47. A lactosylated polylysine nucleic acid complex comprising DNA encoding CFTR, or a biologically active fragment thereof, and lactosylated polylysine, wherein about 10% to about 60% of the amino groups of said polylysine have a lactose molecule substituted thereon, and the weight to weight ratio of lactosylated polylysine to said DNA in said complex is about one to one to about fifteen to one, said complex being capable of transfecting airway epithelial cells  
30 when added thereto.

33. The pharmaceutical composition of claim 32, wherein said glycosylated polylysine comprises lactosylated polylysine.

5 34. The pharmaceutical composition of claim 33, wherein about 10% to about 60% of the amino groups of said polylysine have a lactose molecule substituted thereon.

35. The pharmaceutical composition of claim 34, wherein about 12% to about 40% of the amino groups of said polylysine have a lactose molecule substituted thereon.

10

36. The pharmaceutical composition of claim 33, wherein the weight to weight ratio of lactosylated polylysine to DNA in said complex is about one to one to about fifteen to one.

15

37. A pharmaceutical composition for treatment of a respiratory disease in a human comprising a complex comprising an isolated nucleic acid and a glycosylated polylysine, wherein said isolated nucleic acid comprises antisense DNA capable of inhibiting the expression of a gene, which gene is required for the development of a respiratory disease in a mammal.

20

38. The pharmaceutical composition of claim 37, wherein said gene is selected from the group consisting of an interleukin gene and a gene affecting leukotriene synthesis.

25

39. The pharmaceutical composition of 37, wherein said respiratory disease is asthma and said gene is a gene encoding IL-4.

40. The pharmaceutical composition of claim 37, wherein said respiratory disease is asthma and said gene is a gene encoding IL-5.

30

26. The method of claim 23, wherein said respiratory disease is asthma and said gene is a gene encoding IL-5.

27. A pharmaceutical composition for treatment of a respiratory  
5 disease in a human, comprising a complex comprising an isolated nucleic acid encoding a protein, or a biologically active fragment thereof, and a glycosylated polylysine, wherein said isolated nucleic acid is DNA selected from the group consisting of DNA encoding CFTR, an asthma gene, DNA encoding  $\alpha$ 1 AT, a gene affecting idiopathic pulmonary fibrosis, DNA encoding SP-B and DNA encoding  
10 SP-C, said complex being suspended in a pharmaceutically acceptable carrier, said complex being capable of transfecting airway epithelial cells when added thereto.

28. The pharmaceutical composition of claim 27, further comprising  
15 at least one of chloroquine, glycerol and a fusogenic peptide.

29. The pharmaceutical composition of claim 27, wherein said  
respiratory disease is selected from the group consisting of cystic fibrosis, asthma, emphysema, idiopathic pulmonary fibrosis and congenital deficiency of surfactant  
20 protein.

30. The pharmaceutical composition of claim 27, wherein said DNA  
comprises cDNA.

31. The pharmaceutical composition of claim 30, wherein said  
25 cDNA encodes CFTR.

32. The pharmaceutical composition of claim 27, said glycosylated  
polylysine having a sugar component selected from the group consisting of lactose,  $\alpha$ -glucose,  $\beta$ -galactose, mannose, mannose-6-phosphate, fucose and N-  
30 acetylglucosamine.

18. The method of claim 14, wherein said isolated nucleic acid is DNA.

19. The method of claim 18, wherein said DNA is cDNA.

20. The method of claim 18, wherein said DNA is selected from the group consisting of DNA encoding CFTR, an asthma gene, DNA encoding  $\alpha$ 1AT, a gene affecting idiopathic pulmonary fibrosis, DNA encoding SP-B and DNA encoding SP-C.

21. The method of claim 20, wherein said DNA encodes CFTR.

22. A method of transfecting airway epithelial cells in vivo, said method comprising generating a composition comprising a complex comprising an isolated nucleic acid and a glycosylated polylysine, and adding said complex to said airway epithelial cells.

23. A method of transfecting airway epithelial cells comprising generating a composition comprising a complex comprising an isolated nucleic acid and a glycosylated polylysine, wherein said isolated nucleic acid is antisense DNA capable of inhibiting the expression of a gene, which gene is required for the development of a respiratory disease in a mammal, and adding said complex to said cells.

24. The method of claim 23, wherein said gene is selected from the group consisting of an interleukin gene and a gene affecting leukotriene synthesis.

25. The method of claim 23, wherein said respiratory disease is asthma and said gene is a gene encoding IL-4.

10. A method of transfecting airway epithelial cells comprising adding to said cells a composition comprising a complex comprising an isolated nucleic acid and a glycosylated polylysine, wherein said isolated nucleic acid is antisense DNA capable of inhibiting the expression of a gene, which gene is  
5 required for the development of a respiratory disease in a mammal.

11. The method of claim 10, wherein said gene is selected from the group consisting of an interleukin gene and a gene affecting leukotriene synthesis.

10 12. The method of claim 11, wherein said respiratory disease is asthma and said gene is a gene encoding IL-4.

13. The method of claim 11, wherein said respiratory disease is asthma and said gene is a gene encoding IL-5.  
15

14. A method of transfecting airway epithelial cells, said method comprising generating a composition comprising a complex comprising an isolated nucleic acid and a glycosylated polylysine and at least one of chloroquine, glycerol and a fusogenic peptide, and adding said composition to said airway epithelial cells.  
20

15. The method of claim 14, said glycosylated polylysine having a sugar component selected from the group consisting of lactose,  $\alpha$ -glucose,  $\beta$ -galactose, mannose, mannose-6-phosphate, fucose and N-acetylglucosamine.

25 16. The method of claim 15, wherein said glycosylated polylysine is lactosylated polylysine.

17. The method of claim 14, wherein said cells are transfected *in vitro*.  
30

What is claimed is:

1. A method of transfecting airway epithelial cells comprising adding to said cells a composition comprising a complex comprising an isolated  
5 nucleic acid and a glycosylated polylysine and at least one of chloroquine, glycerol and a fusogenic peptide.
2. The method of claim 1, said glycosylated polylysine having a sugar component selected from the group consisting of lactose,  $\alpha$ -glucose,  $\beta$ -  
10 galactose, mannose, mannose-6-phosphate, fucose and N-acetylglucosamine.
3. The method of claim 2, wherein said glycosylated polylysine is lactosylated polylysine.
4. The method of claim 1, wherein said cells are transfected *in vitro*.  
15
5. The method of claim 1, wherein said isolated nucleic acid is DNA.  
20
6. The method of claim 5, wherein said DNA is cDNA.
7. The method of claim 5, wherein said DNA is selected from the group consisting of DNA encoding CFTR, an asthma gene, DNA encoding  $\alpha$ 1AT, a gene affecting idiopathic pulmonary fibrosis, DNA encoding SP-B and DNA  
25 encoding SP-C.
8. The method of claim 7, wherein said DNA encodes CFTR.
9. A method of transfecting airway epithelial cells *in vivo*  
30 comprising adding to said cells a composition comprising a complex comprising an isolated nucleic acid and a glycosylated polylysine

## Assessment of *In Vivo* Correction of the CF Defect in Animal

### Models

The tracheal xenograft model may be used for these studies. To assess correction of the CF defect *in vivo*, immune incompetent mice which have  
5 been given a denuded rat-trachea transplant under the skin on which CF airway epithelial cells are grafted are used. Aliquots of 100  $\mu$ l of plasmid/polylysine complex plus 50  $\mu$ M chloroquine are administered to the animals at the site of the xenograft. After about 4 hours, the mixture is removed and correction of the CF-associated transepithelial potential difference with amiloride stimulation is  
10 measured after 36-40 hours post transfection.

Alternatively, any of the other animals discussed herein may also be used for administration of CFTR.

As discussed herein, the invention should also be construed to include treatment of CF *in utero* using lactose substituted polylysine DNA  
15 complexes.

The disclosures of each and every patent, patent application and publication cited herein are hereby incorporated herein by reference in their entirety.

While this invention has been disclosed with reference to specific  
20 embodiments, it is apparent that other embodiments and variations of this invention may be devised by others skilled in the art without departing from the true spirit and scope of the invention. The appended claims are intended to be construed to include all such embodiments and equivalent variations.

## Transfer of Reporter Genes into Airway Epithelial Cells in

### Animals

Adult C57/BL6 mice provide an effective animal model for testing the efficacy of glycosylated polylysine vehicles for gene transfer into respiratory epithelial cells *in vivo*. Each of the glycosylated polylysines described herein may be examined in this model for the ability to efficiently transfer DNA into airway epithelial cells *in vivo* as follows.

The glycosylated polylysine is combined with the reporter plasmid CMV-LacZ (CAYLA or other supplier) as described. Aliquots of 100  $\mu$ l containing 2-10  $\mu$ g of plasmid are administered to anesthetized mice via the intranasal or intratracheal route. This method is successful when adenoviral vectors are used and results in reliable gene expression in distal airway and parenchymal cells. One, three, or six doses are administered to the animals intranasally on sequential days. The animals are sacrificed two days following the last dose and are processed for histopathology and  $\beta$ -galactosidase expression. Controls may include recombinant adenoviral vectors (AdCMVLacZ/sub360) to assess cell specificity and distribution of transgene expression (St. George *et al.*, 1995, "Efficacy of Adenoviral Vectors in Airway Epithelium" Cystic Fibrosis Conference, Abstract #151). Newborn lung and fetal lungs from animals may also be tested using the methods described herein.

20

### Transfer of the CFTR Gene into Animals.

CFTR-encoding plasmids are complexed with glycosylated polylysine and are administered to an animal following the procedures just described for transfer of reporter plasmids to animal airway epithelial cells. Expression of CFTR is detected as described herein. Administration of lactose substituted polylysine to the airway cells of animals may be accomplished by aerosol through the nasal passages, by bronchoscopy or by any other method available in the art, such as by tracheal catheter.

In addition to mice, rabbits and other vertebrate animals may be used, including non-human primate animals, to examine the introduction of CFTR into the airway epithelial cells of these animals using the methods described herein.

30



(A) Measurement of  $^{125}\text{I}$ -efflux from CF/T43 cells (Figure 15).  $^{125}\text{I}$ -efflux from cultured CF cells is a measure of whether the CFTR defect in cells has been corrected, wherein correction of the defect results in an increase in efflux of  $^{125}\text{I}$ .  $^{125}\text{I}$ -efflux is measured in a manner similar to that described by Marks *et al.* (1988, *10th International CF Congress*, Sydney, Australia). Essentially, immortalized CF airway epithelial cells (CF/T43) and primary CF and non-CF airway epithelial cells are transfected with the lactosylated polylysine/CFTR plasmid complex using the transfection conditions described herein. The cells are seeded in 35 mm dishes at a cell concentration of  $10^6$  cells per dish. Chloride channel activity in the presence of additives such as forskolin, is assayed by measuring the rate of  $^{125}\text{I}$ -efflux (Venglarik *et al.*, 1990, *Am. J. Physiol.* **259**:C358-C364; Santos *et al.*, 1994, *Biochim. Biophys. Acta* **1195**:96-102; Drumm *et al.*, 1990, *Cell* **62**:1227-1233).

(B) Measurement of SPQ fluorescence. Immortalized CF airway epithelial cells (CF/T43) and primary CF and non-CF airway epithelial cells are cultured on glass coverslips and are transfected with the lactosylated polylysine/CFTR plasmid complex as described herein. Following transfection, the cells are further incubated for 36-48 hours. The cells are then washed with PBS and the halide sensitive fluorophore 6-methoxy-N-(3-sulphopropyl)quinolinium (SPQ) is added. The cells are analyzed according to the method of Yang *et al.* (1993, *Hum. Mol. Gen.* **8**:1253-1261). SPQ fluorescence is quantitated and any increase in fluorescence is an indication that the CFTR defect in the cells has been corrected.

The results of experiments designed to measure CFTR activity by assessing the  $\text{Cl}^-$  activity of the protein by  $^{125}\text{I}$  efflux in the presence of the described stimulants, are presented in Figure 15. In this experiment, T-84 cells expressing cells were examined and exhibited a  $\text{Cl}^-$  efflux rate per minute of 3-4 (Figure 15, Panel A). CF/T1 cells whose defect was corrected by the addition of wild type CFTR, also exhibited an efflux rate which was similar to the response of T-84 cells (Figure 15, Panel B). As expected, CF/T43 cells exhibited no response to the stimulation mixture.

Several other immortalized cell lines were examined to determine the effect of the potentiating agents in increasing the efficacy of transfection of cells. The cells used comprised BEAS2B cells which are immortalized epithelial cells obtained from a non-CF patient (Reddell et al., 1988, Cancer Res. 48:1904-1909). CF/T1 (wild type) immortalized airway cells were also used. These cells were obtained from a CF patient having the  $\Delta F508$  mutation which had been previously transfected with wild type CFTR (Olsen et al., 1992, Humam Gene Therapy 3:253-266). Both of these cells were transfected as described herein in the presence or absence of various additives as indicated in Figure 18. In each case, the cells responded to the additives in a similar manner wherein 50 or 100  $\mu M$  of chloroquine and 5% glycerol yielded the greatest increase in transfection efficiency as measured by expression of luciferase in transfected cells.

The data presented herein establish that the invention can be construed to include transfection mixtures having additional compounds added thereto, which compounds serve to enhance the transfection efficiency of the desired cells. Glycerol or a fusogenic peptide may be added to the transfection mixture, either before, concurrently or following addition of the DNA to the cells.

#### **Examination of the Efficiency of Gene Expression**

In experiments designed to examine the efficiency of gene expression, the reporter gene, pCMVLacZ complexed to lactosylated polylysine was used. Cells were transfected with the reporter gene and expression of the gene was assessed by staining the cells with X-gal. The results of a typical experiment of this type are presented in Figure 16. Approximately 40% of the cells exhibited intense blue staining. After three subsequent transfections, 90% of the cells exhibited moderate to intense blue staining which was not evident in control untransfected cells. These experiments establish that CF/T43 cells may be transfected with high efficiency using the composition of the invention as described herein.

#### **Assessment of Whether Transfection of the *CFTR* Gene Results in Functional Correction of the CF Defect in Airway Epithelial Cells**

polylysine should serve to enhance transfection of airway epithelial cells in a safe and effective manner.

The amount of chloroquine to be included in the transfection mixture for administration to humans or animals may vary from about 25  $\mu\text{M}$  to about 200  $\mu\text{M}$  per dose. Preferably, the amount of chloroquine to be included in the mixture is from about 50  $\mu\text{M}$  to about 100  $\mu\text{M}$ .

Other enhancing agents may be used in the transfection mixture in place of or in addition to chloroquine. Such agents include, but are not limited to, bioactive peptides and glycerol. Suitable peptides include, but are not limited to, E5CA-GLFEAIAEFIEGGWEGLIEGCA (Midoux *et al.*, 1993, *Nucleic Acids Res.* 21:871-878), HA-2-GLFEAIAAGFIENGWEGMIDGGGC (Wagner *et al.*, 1992, *Proc. Natl. Acad. Sci. USA* 89:7934-7938) and JTS-1-GLFEALLELLESLWELLLEA (Gottshalk *et al.*, 1996, *Gene Ther.* 3:448-457).

Examples of the enhancement of transfection by glycerol and the fusogenic peptide E5CA are now described.

Cells were transfected with pCMVLuc complexed to lactosylated polylysine in the presence or absence of glycerol and/or chloroquine. Following a four hour incubation period with the transfection mixture, the cells were treated as described herein and were incubated for a further 48 hours. The cells were then lysed and luciferase activity expressed therein was assessed as described herein. It is evident from the data shown in Figures 10 and 11 that glycerol enhances transfection of both CF/T43 cells and primary airway epithelial cells when the cells are transfected either with or without chloroquine.

In a similar manner to that just described, CF/T43 cells were transfected for three hours with a reporter gene complexed to gluconoylated polylysine in the presence of increasing amounts of the fusogenic peptide, E5CA. The results of these experiments are shown in Figure 12. It is evident that the transfection efficiency of the cells was markedly enhanced in the presence of fusogenic peptide. The enhancement of luciferase gene expression in CF/T43 cells transfected with a lactosylated polylysine DNA complex is summarized in Figure 13.

expression of *CFTR* in transfected cells may be assessed by measuring the amount of *CFTR* protein in the cells using any one of the several methods as described herein.

Western blotting analysis may be performed using antibody to the R domain of *CFTR* as described in Wei *et al.* (1996, *J. Cell. Physiol.* **168**:373-384) and available technology described for example in Sambrook *et al.* (Molecular Cloning: A Laboratory Manual, Cold Spring Harbor, NY, 1989).

Antibodies are prepared by synthesizing peptides from the deduced amino acid sequence of the R domain and the first ATP binding fold. Peptides may be obtained preferably from regions which are highly conserved between the bovine and human *CFTR* sequences (Diamond *et al.*, 1991, *J. Biol. Chem.* **266**:22761-22769).

To obtain antibody directed against the R domain of *CFTR*, peptides including that domain are covalently coupled to tuberculin purified protein derivative (PPD) and are then inoculated into rabbits. Antibody is then purified from serum obtained from the rabbits at periodic intervals. The technology for making antibodies directed against specific peptides is well known in the art and is described for example, in Harlow *et al.* (1988, *In: Antibodies, A Laboratory Manual*, Cold Spring Harbor, NY). Antibodies to the C terminus domain of *CFTR* may also be used (Mulberg *et al.*, 1994, *Neuroreport* **5**:1684-1688).

Alternatively, expression of *CFTR* may be assessed using a plasmid containing *CFTR* having a M2-901 epitope tag (Howard *et al.*, 1995, *Am J. Physiol.* **269**:1565-1576). Protein expressed by this plasmid may be detected using M2 antibody which is commercially available (Eastman Kodak Co.) and the methods described by Howard *et al.* (*supra*).

The invention further includes administration of chloroquine or other enhancing agent in combination with lactose substituted polylysine *CFTR* DNA complexes to patients having CF. Administration of chloroquine to humans is known for the prevention of malaria infection. As described herein, chloroquine enhances DNA transfer of sugar substituted polylysine DNA complexes into cells. Thus, administration of this compound in combination with DNA and glycosylated

about 90%. The highest transfection efficiency in these cells was accomplished using 5% glycerol and 10 µg of fusogenic peptide in the transfection medium. As shown in Table 1, other combinations of potentiating agents were also used to successfully transfect these cells.

5

**TABLE 1**

In situ hybridization of CF cells in primary culture to detect CFTR mRNA after transfection with pAdCFTR complexed to lactosylated polylysine

10

<u>Cells<sup>a</sup></u>	<u>CFTR Expressing</u>		
	<u>High</u>	<u>Medium</u>	<u>Transfected</u>
<u>Additives</u>			
<u>counted</u>			<u>Percentage of total</u>
None	22	58	80
glycerol + Fusogenic peptide <sup>b</sup>	67	22	89
Glycerol	44	56	100
Chloroquine + Fusogenic peptide	31	68	99
Chloroquine	23	66	89
Plasmid	0	10	--
<u>Three Additions</u>			
Glycerol + Fusogenic peptide <sup>b</sup>	30	65	95
Glycerol <sup>b</sup>	35	54	94
Chloroquine + Fusogenic peptide	34	62	91

15

20

25

<sup>a</sup> Based on the number of intensely blue (high) or moderately blue (medium) cells.

30

<sup>b</sup> Average of 2 separate experiments which deviated by 10% or less.

The plasmid/polylysine complex transfected cells may be added to airway epithelial cells as described herein. In addition to assessing expression of *CFTR* by measuring CFTR-specific mRNA using *in situ* hybridization, the

In summary, the data described herein establish that polylysine which is partially substituted with either glycosyl or gluconoyl residues is an effective non-viral vehicle for transfer of DNA into airway epithelial cells. However, glycosylated polylysine, and in particular lactosylated polylysine, is most effective in mediating gene transfer into airway epithelial cells. The presence of chloroquine or other additives as described herein, in the transfection medium enhanced gene transfer in airway epithelial cells even in primary cell cultures.

Thus, according to the present invention, a novel method of transfer of the CF gene into airway epithelial cells has been discovered, which method is useful for treatment of CF. As noted herein, glycosylated polylysines are largely non-immunogenic; thus, they are superior to other non-viral vehicles and are vastly superior to viral vectors as a gene therapy approach for treatment of CF.

#### **Transfer of the *CFTR* Gene into Primary Cells in Culture Using Lactosylated Polylysine as a Delivery Vector**

The technique of *in situ* hybridization was used to demonstrate transfer of the *CFTR* gene into the immortalized cell line, CF/T43, and into cells in primary culture. An example of the results of this type of experiment is shown in Figure 17. For these studies, cells were grown on coverslips and were transfected with 3  $\mu$ g of the plasmid, pAd*CFTR*, or the plasmid, pBQ*CFTR* (Drumm et al., 1990, Cell 62:1227-1233) complexed to 9  $\mu$ g of lactosylated polylysine. Cells were transfected on three consecutive days in the presence of 5% glycerol and 10  $\mu$ g fusogenic peptide, in the case of the primary cells, and in the presence of 100  $\mu$ M chloroquine and 5% glycerol, in the case of the immortalized cells. Other combinations of potentiating agents were also used (Table 1). Expression of *CFTR* was detected by *in situ* hybridization using exon 14 of *CFTR* labeled with deoxygenin as a probe. Labeled or unlabeled cells were visualized in a Nikon Diaphot 300 microscope and the amount of label was quantitated therein.

A variety of control cells, including plasmid which was not complexed with lactosylated polylysine, exhibited about a 25% transfection efficiency. In contrast to the control cells, the transfection efficiency of primary cultures of cells obtained from nasal polyps or trachea was between about 70 and

lactosyl polylysine had decreased, likely due to the fragility of these particular cells and their response to the presence of chloroquine in the transfection medium.

The concentration of chloroquine required to induce maximum transfection efficiency in human non-CF airway epithelial cells was observed to be lower than that observed in CF/T43 cells (compare Figures 5C and Figure 8). An optimal level of luciferase expression was observed in primary cells in the presence of 50  $\mu$ M chloroquine.

In summary, the results just described establish that primary cell cultures (both CF and non-CF) may be efficiently transfected with DNA using glycosylated polylysine as a transfection vehicle. Lactosylated polylysine is superior to other sugar substitutions and gluconoylated polylysine is an inefficient vehicle for gene transfer in these cells.

#### **Endogenous Lectins on CF/T43 Cells Which Bind**

##### **Lactose**

To assess whether the observed enhanced transfection efficiency using lactosylated polylysine is related to any direct interaction between lactosylated polylysine and the carbohydrate binding proteins on the outer surface of the cells, binding studies were conducted. In particular, the binding of lactose-neoglycoprotein was examined.

Lactose-neoglycoprotein was prepared by conjugating lactose to BSA-FITC. This compound was added to CF/T43 cells in a binding assay and was observed to bind to the surface of these cells at 4°C (Figure 9A). Since binding was blocked by the addition of 0.1 M or 0.2 M lactose, the observed binding was considered to be a specific rather than a non-specific binding of lactose to the cell surface (Figure 9B). FITC-labeled-BSA did not bind under these conditions. These experiments therefore demonstrate the presence of endogenous lactose-binding lectins on the surface of the airway cells.

In additional experiments, increasing concentrations of lactose added to the transfection medium were observed to effect a decrease in the expression of luciferase. When lactose was added to the transfection medium at a concentration of 0.1 M, a 60 % decrease in luciferase expression was observed.

The presence of chloroquine in the transfection mixture containing lactosylated polylysine also resulted in an overall increase in expression of luciferase (Figure 5C). When chloroquine was absent, luciferase activity was observed to be only 2% of the level observed when 100  $\mu$ M of chloroquine was added to the transfection mixture.

#### **Transfection of Primary CF Airway Epithelial Cells in Culture**

CF airway epithelial cells grown from tracheal tissue explants were transfected and examined for the expression of luciferase using the glycosylated or gluconoylated substituted polylysines as the vehicle and pCMV*Luc* as the expression plasmid. The results of experiments conducted in two different primary cultures are shown in Figure 6. A high level of luciferase gene expression was observed when lactosylated polylysine served as the transfection vehicle. Other glycosylated polylysines resulted in approximately 10-fold less luciferase activity in transfected CF cells, and in the case of Fuc-substituted polylysine, luciferase activity was 80-fold less than that observed using lactosylated polylysine.

#### **The Use of Glycosylated Polylysines as Vehicles for Transfer of DNA into Primary Human (non-CF) Airway Epithelial Cells**

Tissue explants were obtained from tracheal tissue which was itself obtained from lung transplant patients. Airway epithelial cells were grown from the tissue explants and were transfected as described herein using a variety of sugar substituted polylysines. These results are shown in Figure 7.

Consistent with the results described above, lactosylated polylysine was an effective vehicle for transfection of non-CF airway epithelial cells (Figure 7A).  $\alpha$ -Glucose- or  $\beta$ -galactose-substituted polylysines were also effective vehicles. In another experiment, galactosylated polylysine proved to be less than 50% as effective for transfection compared with lactosylated polylysine (Figure 7B). Non-substituted polylysine was only 8% as effective as lactosylated polylysine.

When the time of transfection was increased from 4 to 6 hours, the expression of luciferase was increased by 40% when lactosylated polylysine was used as the transfection vehicle. However, by 8 hours, the transfection efficiency of



expression when used to transfect cells. The levels of luciferase activity ranged from 3.9 to 0.3 X 10<sup>8</sup> RLU/mg protein in the following order of polylysine complexes used in the transfection mixtures:  $\beta$ -Gal =  $\alpha$ -Glc = Lac >  $\alpha$ -Gal = Rha = Man >  $\beta$ -GalNAc > GlcA >  $\alpha$ -GalNAc =  $\alpha$ -Fuc.

5 In separate experiments, GlcNAc-substituted polylysine was not as efficient a vehicle as lactosylated polylysine; GlcNAc-substituted polylysine transfected cells exhibited less than 50% of the luciferase activity observed in cells transfected with lactosylated polylysine. Further, non-substituted polylysine was only 10% as effective as lactosylated polylysine in effecting expression of  
10 luciferase in transfected cells. Thus,  $\alpha$ -glucose,  $\beta$ -galactose and lactose substituted polylysine are superior to other sugar substitutions on polylysine for transfer of DNA into immortalized airway epithelial cells.

#### **Optimal Conditions for Lactosylated Polylysine Mediated Gene Transfer**

15 The data presented in Figure 4 establish that lactosylated polylysine is a highly efficient vehicle for the transfection of CF/T43 cells. To determine the optimal conditions for use of lactosylated polylysine as a transfection vehicle in CF/T43 cells, the following experiments were conducted.

The ratio (w/w) of the lactosylated polylysine to pCMV*Luc* was  
20 varied to determine the optimum ratio for efficient transfection of plasmid. These results are shown in Figure 5A. When a 4:1 ratio was used in the transfection mixture, the expression of luciferase in transfected cells was increased two-fold, compared with a 3:1 ratio.

The effect of time on transfection efficiency was also determined.  
25 When the time of transfection was increased to 8 hours from 4 hours, luciferase activity was increased in transfected CF/T43 cells (Figure 5B). However, at a transfection time of 8 hours, some morphological changes were observed in the cells. The optimal time of transfection using lactosylated polylysine in the presence of 100  $\mu$ M chloroquine was approximately 6 hours. In these cells, expression of  
30 luciferase continued for up to 120 hours.

time post transfection, the cells were harvested and luciferase activity was measured (Figure 3B). Maximal luciferase activity was observed between 48 and 96 hours post transfection. In fact, when the time was extended to 120 hours post transfection, a very high level of luciferase activity (130% of that observed at 48 hours post transfection) was observed. Since during the course of this latter experiment the cells were incubated in growth medium, the cell number actually increased, therefore apparently lowering the amount of luciferase activity when measured as activity per mg of protein.

### The Use of Glycosylated Polylysines for Transfection of CF/T43

#### 10 Cells

Glycosylated polylysines containing varying numbers of mono or disaccharides were prepared as described herein. Polylysine substituted with monosaccharides contained an average of  $77 \pm 10$  sugar residues corresponding to the substitution of  $41 \pm 5\%$  of the amino groups of polylysine (DP-190). Lactosylated polylysine contained an average of 66 lactose residues corresponding to the substitution of 34% of the amino groups.

Glycosylated polylysine/plasmid complexes were made having the lowest polymer to DNA ratio (2 or 2.5  $\mu\text{g}$  polylysine substituted with monosaccharides per  $\mu\text{g}$  pCMV*Luc* and 3  $\mu\text{g}$  lactosylated polylysine per  $\mu\text{g}$  pCMV*Luc*). At these concentrations and ratios the following was observed: (i) a complete retardation of all the DNA during electrophoresis; (ii) complexes which had a pH near 7.0; (iii) a complete association of glycosylated polylysine with the DNA; and iv) high efficiency gene transfer.

When transfection mixtures were prepared in this manner, the level of luciferase activity was observed to vary with the type carbohydrate contained on the polylysine (Figure 4). The use of polylysine substituted with  $\beta$ -Gal,  $\alpha$ -Glc or lactose complexed with pCMV*Luc* yielded luciferase activity at levels of 322%, 298% and 289%, respectively, when compared with the use of gluconoylated polylysine. Polylysine substituted with Rha, Man and  $\alpha$ -Gal also yielded high expression of luciferase activity; however, several other carbohydrate substitutions, namely  $\alpha$ -L-Fuc and  $\alpha$ -GalNAc yielded negligible amounts of luciferase gene

the lowest possible molar ratio between the vehicle and DNA. In other words, the ratio of polylysine to DNA was adjusted so that the transfection medium did not contain any DNA-free vehicle which might otherwise contribute to cellular toxicity (Erbacher *et al.*, 1995, *supra*). When the vehicle:plasmid ratio was 2:1 there were  
5 no observed morphological changes in the cells. When non-substituted polylysine was used as a vehicle complexed with pCMV*Luc* at a ratio of 2:1 (w/w), luciferase was expressed to a level of only 40% of that observed using gluconoylated polylysine.

To assess the effect of cell number on transfection efficiency and  
10 gene expression, the number of CF/T43 cells in each transfection assay was varied while maintaining the plasmid concentration at 1  $\mu$ g and the ratio of gluconoylated polylysine to DNA at 2:1 (w/w). In the majority of the experiments, CF/T43 cells were seeded at a concentration of  $1.5 \times 10^5$  cells per 25 mm well. However, when  
15  $2 \times 10^5$  cells per well were used, a higher level of luciferase activity was observed (Figure 2B). When the cell concentration was increased to greater than  $2 \times 10^5$  cells per well, no additional increase in luciferase activity was observed. Thus, the number of cells seeded in each well has an effect on the transfection efficiency of the cells.

The effect of the length of time of transfection on transfection  
20 efficiency and gene expression was assessed as follows. DNA was added to cells in a standard transfection mixture at a gluconoylated polylysine to DNA ratio of 2:1. Cells were incubated with the DNA transfection mixture for 4 to 10 hours in the presence of 100  $\mu$ M chloroquine. At each time point tested, the transfection mixture was removed from the cells, the cells were washed and incubated in fresh  
25 medium for 48 hours prior to lysis and luciferase assay. From Figure 3A it is evident that the amount of luciferase activity increased about 4-fold in transfected CF/T43 cells when the transfection time was increased from 4 to 10 hours. In another experiment using 5  $\mu$ g of plasmid, luciferase activity was observed to peak a transfection time of 6 hours.

30 To assess the effects of incubation time post transfection on gene expression in transfected cells, cells were transfected for 4 hours, and at various

Controls included cells transfected with either of the plasmids or the vehicles on their own.

### **Optimal conditions for Gene Transfer Using Gluconoylated Polylysine**

5 A series of experiments were performed to determine the optimum conditions for the expression of the luciferase gene following transfection of a luciferase encoding plasmid into CF/T43 cells.

To assess the effects of chloroquine on transfection, cells were transfected in the presence of increasing concentrations of this compound. The use  
10 of chloroquine in the transfection mixture resulted in an increase in the transfection efficiency of CF/T43 cells by the gluconoylated polylysine/plasmid complex in a concentration-dependent manner (Figure 1). The amount of luciferase activity expressed in cells transfected with a chloroquine-containing mixture was 75-fold higher than in cells transfected in the absence of chloroquine. An approximately  
15 26-fold increase in luciferase activity was observed when 100  $\mu$ M chloroquine was used compared with the level of luciferase activity in cells transfected in the presence of 10  $\mu$ M chloroquine. When 200  $\mu$ M chloroquine was used the level of luciferase activity was significantly decreased in transfected cells. Fetal bovine serum, at a concentration of 1-10%, had little effect on the transfection efficiency  
20 during a 4 hour transfection time period. Thus, the concentration of chloroquine used affected the transfection efficiency and the highest level of reporter gene expression was observed at a chloroquine concentration of 100  $\mu$ M.

To assess the effect of plasmid concentration on transfection efficiency and gene expression, the concentration of the plasmid pCMV*Luc* (pUT  
25 650) was varied from 0.1 to 10  $\mu$ g, while maintaining the gluconoylated polylysine:plasmid ratio at 2:1 (Figure 2A). When the concentration of the plasmid complex was linearly increased, luciferase activity also increased linearly. A concentration of 1  $\mu$ g of pCMV*Luc* was used in subsequent experiments, unless otherwise stated.

30 Increasing amounts of plasmid did not result in increased toxicity to cells because gluconoylated polylysine/plasmid complexes were designed to have

In experiments designed to measure the efficiency of gene expression, the plasmid pCMVLacZ may also be used, wherein gene expression is measured by measuring  $\beta$ -galactosidase activity using the chromogenic reagent X-gal.

5

#### **Binding of FITC Neoglycoproteins**

Bovine serum albumin (BSA) was lactosylated and subsequently labeled with fluorescein-conjugated isothiocyanate (FITC) as described (Monsigny *et al.*, 1984, *supra*). CF/T43 cells (100-200 cells) were grown on cover slips for 2 days and after removal of the growth medium the cells were incubated with 20-100  $\mu$ g per ml of either lactose-BSA-FITC or BSA-FITC at 4°C for 30 minutes. In some cases, 0.1 or 0.2 M lactose was added with the neoglycoprotein to block binding. The coverslips were washed three times at 4°C with phosphate buffered saline, pH 7.3, and fixed in methanol for 10 minutes at 4°C. After mounting with SlowFade™-light Antifade Kit (Molecular Probes Inc.), binding was assessed using a Nikon Diaphot 300 microscope.

15

#### **The Use of Gluconoylated Polylysine as a Transfection Vehicle in CF/T43 Cells**

To establish some optimum parameters for transfection of cells using glycosylated polylysines, gluconoylated polylysines were initially examined in the transfection assays described herein. Seventy-four amino groups of polylysine (DP 190) were substituted with gluconoyl residues by acylation with  $\delta$ -gluconolactone. This substitution provided a partially neutralized derivative of polylysine which was highly water-soluble and an efficient vehicle for transfecting various cell lines (Midoux *et al.*, WO 95/30020). Gluconoylated polylysine was an efficient vehicle for the transfer of several luciferase plasmids into CF/T43 cells, resulting in high levels of gene expression in these cells.

20

25

Two different luciferase encoding plasmids, wherein the luciferase was placed under the control of two different promoters (pCMVLuc or pSV2Luc) were used. Either of these plasmids was complexed to gluconoylated polylysine at a 2 to 1 (w/w) ratio of polylysine to DNA. The plasmid pCMVLuc was 26 times more effective than pSV2Luc for the expression of luciferase in CF/T43 cells.

30

the cells were transfected daily for three days. The use of this protocol permits the use of larger quantities of vector/plasmid and this protocol is therefore useful for *in vivo* administration of DNA when it is necessary to maintain higher constant levels of expressed protein products.

5 To detect luciferase gene expression in transfected cells, the cells from each well were lysed by the addition of 100  $\mu$ l of cell culture lysis reagent (25 mM Tris, pH 7.8; 2 mM EDTA; 2 mM DTT; 10% glycerol; 1% Triton X-100). The cell lysate was incubated for 15 minutes at ambient temperature and was transferred to Eppendorf tubes for centrifugation.

#### 10 Measurement of Luciferase Activity

Luciferase activity was assessed by measuring luminescence following the method of De Wet *et al.* (1987, *Mol. Cell. Biol.* 7:725-737). The assay mixture (270  $\mu$ M coenzyme A, 470  $\mu$ M luciferin, 530  $\mu$ M ATP, 20 mM tricine, 1.07 mM  $(\text{MgCO}_3)_4$   $\text{Mg}(\text{OH})_2 \cdot 5\text{H}_2\text{O}$ , 2.67 mM  $\text{MgSO}_4$ , 0.1 mM EDTA, 15 33.3 mM DTT, pH 7.8) was added at a ratio of cell lysate:assay mixture of 1:4, which was determined to be the optimum ratio for luciferase activity. Luminescence was recorded on a Luminat luminometer, LB 9501 (EG&G Berthold Analytical Instruments Inc.) for 5 sec and reported as relative light units (RLU). The luciferin/luciferase assay system was stable for more than 60 sec and duplicate 20 wells were assayed in amounts of 5 to 20  $\mu$ l to assure linearity of the results. Different experiments were performed and each was repeated twice. The standard assay points within experiments have a mean RLU of  $3.7 \times 10^7 \pm 2.8 \times 10^6$  SEM (standard error of the mean) (n=11) and  $1.3 \times 10^8 \pm 2.1 \times 10^7$  SEM (n=8) per mg of protein when gluconoylated or lactosylated polylysine, respectively, served as 25 vehicle. A blank assay containing the cell extract but no plasmid, yielded a reading of 120-200 RLU. Protein concentration was determined using the method of Lowry *et al.*, in cells which were lysed with 0.1 M NaOH or in the individual wells containing cells using the lysis buffer as background (Lowry *et al.*, 1951, *J. Biol. Chem.* 193:265-275). The results are expressed as RLU per mg of protein. One 30 picogram of luciferase is equivalent to 11,000 RLU under these assay conditions.

### Transfection Procedures

The polymer/plasmid complexes were prepared as described (Erbacher *et al.*, 1995, *supra*). Gluconoylated or glycosylated polylysine (20-30  $\mu\text{g}$  of polylysine dissolved in 0.3 ml of serum-free DMEM) was added to the reporter plasmids (10  $\mu\text{g}$  of DNA dissolved in 0.7 ml of serum-free DMEM) and the mixture was incubated for 30 minutes at ambient temperature unless otherwise specified. To prevent precipitation of the complex, the glycosylated polylysine was added to the plasmid at a rate of 15  $\mu\text{l}$  per 30 seconds.

The lowest vehicle to DNA ratio (w/w) which exhibited complete retardation of all the DNA during electrophoresis was used. In that instance, all of the DNA was condensed and no free polymer was detected. The ratios were experimentally determined and ranged from 2 to 3. Next, 1 ml of DMEM containing the polymer/plasmid complex was supplemented with 1% heat inactivated fetal bovine serum and 100  $\mu\text{M}$  chloroquine, and this mixture was added to each set of cells after removal of the growth medium from the cells.

After incubation at 37°C for 4 hours unless otherwise specified, the transfection mixture was removed and the cells were further incubated at 37°C in 2.0 ml KGM medium without additives. The cells were examined morphologically both after transfection and prior to lysis. Except when specified, all cells appeared normal. After 48 hours or at other time intervals, the cells were processed and expression of the transfected gene was assessed.

In order to increase the efficiency of transfection, the following protocol was adopted. Lactosylated polylysine (2  $\mu\text{g}$  in 30  $\mu\text{l}$  of DMEM) was added at a rate of 15  $\mu\text{l}$  per 30 to 60 seconds to the appropriate plasmid (1  $\mu\text{g}$  of plasmid dissolved in 70  $\mu\text{l}$  DMEM). The mixture was allowed to sit for an additional 30 minutes at ambient temperature to form a complex. The complex so formed was supplemented as follows: 100  $\mu\text{M}$  chloroquine and either 5% glycerol or 10  $\mu\text{g}$  fusogenic peptide in the case of immortalized cells; and 5% glycerol with or without fusogenic peptide in the case of the primary cells. Each final mixture was contained in 1 ml of DMEM without serum and was then added to the cells. Thereafter, the transfection procedures was as described herein, and where noted,

sugar residues bound per polylysine molecule was calculated from the sugar content determined by the resorcinol sulfuric acid micromethod (Monsigny *et al.*, 1988, *Biochimie* **70**:1633-1649). The 66 lactose residues which bound to polylysine corresponded to a substitution of 35% of the amino groups of the molecule. The  
5 number of monosaccharide residues ( $77 \pm 10$ ) which bound corresponded to a substitution of  $41 \pm 5\%$  of the amino groups of polylysine.

Modification of the above-described procedure yields a compound having even higher activity when complexed to pCMVLuc (Figure 14). These modifications include beginning with 50% less starting material, mixing for 24  
10 hours at 24°C, and washing the precipitate with isopropanol prior to lyophilization. About 20% of the amino groups on the polylysine are substituted with a glycosyl residue following this procedure.

In addition, glycosylated polylysines may be prepared by reductive complexing of lactose to polylysine with cyanoborohydride as described (Matrinez-Fong *et al.*, 1994, *Hepatology* **20**:1602-1608).  
15

### Cell Culture

The immortalized airway epithelial cell line, CF/T43 was obtained from a CF patient homozygous for the  $\Delta F508$  mutation. The cells were grown as described (Jetten *et al.*, 1989, *Science* **244**:1472-1475) in KGM medium.

20 Primary cultures of cells were prepared from tracheal explants or nasal polyps obtained from CF or non-CF patients at the time of surgery. Tracheal pieces were stripped and gently minced and were then placed in 25 cm<sup>2</sup> flasks coated with 25% fibronectin and incubated in LHC-9 medium at 37°C in an atmosphere containing 5% CO<sub>2</sub>. When epithelial cells grew out from the tissue, the  
25 pieces were removed to a new flask until additional epithelial cells grew out. This procedure was repeated several times. In some cases, the cells were obtained by protease treatment of the tissue as described (Wu *et al.*, 1990, *Am. J. Respir. Cell Mol. Biol.* **3**:467-478).

Prior to transfection, cells were seeded at  $1.5 \times 10^5$  cells per 25 mm  
30 well in a 12-well plate (Corning). CF/T43 cells were then incubated for 24 hours at 37°C and the primary cells were incubated from 24-48 hours at 37°C.



CAYLA) was used. Thus, the plasmids pCMV $Luc$  and pUT 650 each contain the luciferase gene placed under the control of the human CMV promoter.

#### Preparation of Gluconoylated Polylysine

Polylysine, HBr (average molecular weight of 40,000; DP-190) obtained from Bachem Feinchemikalien, Bubendorf, Switzerland, was dissolved in H<sub>2</sub>O (1 g in 200 ml) and passed through an anion exchange column (Dowex 2 X 8, OH<sup>-</sup> form, 20-50 mesh, 35 X 2.5 cm) in order to remove bromide ions (Derrien *et al.*, 1989, *Glycoconjugate J.* 6:241-255). The effluent solution was neutralized with 10% *p*-toluene sulfonic acid in water (a non-cytotoxic compound) and was subsequently lyophilized.

Polylysine was partially substituted with gluconoyl residues as described (Derrien *et al.*, *supra*). Briefly,  $\delta$ -gluconolactone (15 mg; Aldrich Chemical Co.) was added to polylysine *p*-toluene sulfonate salt (50 mg) in 3 ml dimethylsulfoxide in the presence of 37  $\mu$ l diisopropylethylamine (Aldrich Chemical Co.); the concentration was adjusted to 1% with water and the solution was stirred for 24 hours at 20°C. Gluconoylated polylysine was precipitated by adding 10 volumes of isopropanol and the precipitate was collected by centrifugation at 1800 X g for 15 minutes. The pellet was washed with isopropanol, collected again by centrifugation, solubilized in distilled water and was lyophilized. The average number of GlcA residues bound per polylysine molecule was determined by <sup>1</sup>H-NMR spectroscopy (300 MHz, D<sub>2</sub>O) and was found to be 74 residues.

#### Preparation of Glycosylated Polylysine Conjugates

Polylysine (DP 190) was partially substituted with sugar residues as described (Midoux *et al.*, 1993, *supra*; Erbacher *et al.*, 1995, *supra*). The 4-isothiocyanatophenyl-derivatives of either  $\beta$ -D-Gal,  $\alpha$ -D-Gal,  $\alpha$ -D-Glc,  $\alpha$ -L-Rha,  $\alpha$ -L-Fuc,  $\alpha$ -D-Man,  $\beta$ -D-GlcNAc,  $\alpha$ -D-GalNAc,  $\beta$ -D-GalNAc or  $\beta$ -D-Lac (Monsigny *et al.*, 1984, *Biol. Cell* 51:187-196) were added to polylysine *p*-toluene sulfonate salt in dimethylsulfoxide in the presence of diisopropylethylamine and the mixture was incubated for 24 hours at 20°C. Glycosylated polylysine was precipitated and processed as described above for gluconoylated polylysine. The average number of

Luciferase activity in these cells was enhanced by 75-fold when the transfection mixture included 100  $\mu$ M chloroquine. Luciferase gene expression persisted at high levels for up to at least 120 hours following transfection.

5 Glycosylated polylysine/pCMV*Luc* complexes were compared with the gluconoylated polylysine/pCMV*Luc* complex in immortalized airway epithelial cells. In some cases, pCMV*LacZ* encoding  $\beta$ -galactosidase was used (Gao *et al.*, 1993, *Human Gene Therapy* 4:17-24). It was found that  $\beta$ -galactose,  $\alpha$ -glucose and lactose-substituted polylysines resulted in 320%, 300% and 290% correspondingly higher levels of expression of the reporter gene luciferase than that expressed from  
10 gluconoylated polylysine complexed with the same DNA. The amount of luciferase expressed following transfection with the test polylysines ranged from 35 to 2 ng of luciferase per mg of cell protein in the following order:  $\beta$ -Gal =  $\alpha$ -Glc = Lac >  $\alpha$ -Gal = Rha = Man >  $\beta$ -GalNAc >  $\alpha$ -GalNAc =  $\alpha$ -Fuc. These results establish that the transfection efficiency of the subject cells is sugar dependent. Importantly,  
15 when primary cultures of either CF or non-CF airway epithelial cells grown from tracheal tissue explants were used, lactosylated polylysine yielded uniformly high expression of luciferase activity.

#### Materials Used in the Studies Described Herein

Lysis and assay buffers for determination of luciferase activity were  
20 obtained from Promega Corp. Luciferase was obtained from Boeringher-Mannheim Corp. Chloroquine was obtained from Sigma Chemical Co. The media used for cell culture included KGM obtained from Clonetics Corp., LHC-9 obtained from Biofluids Inc., and DMEM obtained from Hazelton Laboratories. Fetal bovine serum was obtained from Biofluids Inc.

25 Expression plasmids encoding the firefly luminescence gene were pSV2*Luc* (5 kb) (Brasier *et al.*, 1989, *Biotechniques* 7:1116-1123), and pCMV*Luc*, having 6.2 kb (Erbacher *et al.*, 1995, *supra*).

The plasmid pSV2*Luc* comprises the luciferase gene placed under the control of the SV40 early promoter, while pCMV*Luc* comprises the luciferase  
30 gene placed under the control of the human CMV promoter. When specified, pUT 650 containing a CMV promoter with *Luc::Sh ble* fusion gene (purchased from

as an example and bearing in mind that the use of the kit is in no way limited to this cell type, the kit may be used as follows. Each glycosylated polylysine derivative is individually complexed to a reporter DNA following the procedures described herein. Aliquots of cells are transfected with each complex and expression of the reporter DNA is assessed at selected times post transfection as a measure of transfection efficiency. The glycosylated polylysine derivative yielding the highest efficiency of transfection of the cells will be evident from the levels of expression of the reporter gene in each of the groups of transfected cells. This particular glycosylated polylysine derivative may then be used for all subsequent transfection experiments involving alveolar macrophages. In this way, it is possible to identify a means for high efficiency transfection of any number of different cell types using the kit of the invention.

The unique features of the cell transfection kit of the invention take advantage of the sugar dependent, receptor mediated endocytosis by chemical conjugation of sugars with polylysine which serves to reduce the positive charge of the polylysine while still permitting effective binding with a desired DNA, all DNA's having an overall negative charge which charge facilitates binding of the DNA to compounds such as polylysine. The effectiveness of the method is augmented by the addition of adding glycerol and other known lysosomotropic agents such as chloroquine and fusogenic peptides.

The invention is further described in detail by reference to the following experimental examples. These examples are provided for purposes of illustration only, and are not intended to be limiting unless otherwise specified. Thus, the invention should in no way be construed as being limited to the following examples, but rather, should be construed to encompass any and all variations which become evident as a result of the teaching provided herein.

The results of the experiments described below are now summarized. When gluconoylated polylysine was used as a vehicle, the reporter plasmid pCMV*Luc*, comprising luciferase coding sequences under the control of the human cytomegalovirus (CMV) promoter/regulatory region, yielded a high level of expression of luciferase when transfected into immortalized CF/T43 cells.

Such compounds include glycerol which may be used at a preferred concentration of about 5%, although the use of glycerol at concentrations other than 5% is also contemplated, for example, glycerol concentrations of about 1% to about 8% are contemplated.

5 In addition, it has been discovered in the present invention that fusogenic peptides may be used to enhance the transfection efficiency of glycosylated polysine DNA complexes into cells. Examples of fusogenic peptides which may be included in the cell transfection kit are provided in the experimental details section herein, and include, but are not limited to, E5CA-  
10 GLFEAIAEFIEGGWEGGLIEGCA (Midoux *et al.*, 1993, *Nucleic Acids Res.* **21**:871-878), HA-2-GLFEA1AGFIENGWEGMIDGGGC (Wagner *et al.*, 1992, *Proc. Natl. Acad. Sci. USA* **89**:7934-7938) and JTS-1-GLFEALLESLESLWELLLEA (Gottshalk *et al.*, 1996, *Gene Ther.* **3**:448-457). While the peptides may be used in the transfection mixture at a preferred concentration of about 10  $\mu$ g, other  
15 concentrations of fusogenic peptide may also be used. The concentration of fusogenic peptide useful in the methods of the present invention may vary from about 1  $\mu$ g to about 50  $\mu$ g.

In addition, the kit may further include an amount of chloroquine, preferably to be used at a concentration of about 100  $\mu$ M, although other  
20 concentrations of chloroquine are also contemplated. The concentration of chloroquine which may be used may vary from about 30  $\mu$ M to about 150  $\mu$ M.

As described herein, DNA complexed with lactosylated polylysine may be transfected into either immortalized or primary cultures of airway epithelial cells with high efficiency. While not wishing to be bound by any theory, it is  
25 believed that transfection of these cells by lactosylated polylysine DNA complexes occurs via receptor mediated endocytosis. Since many other cell types also have the capacity for receptor mediated endocytosis, a kit comprising a variety of glycosylated polylysine derivatives for complexing to DNA will enable the transfection of a number of different cell types. Use of the kit will also enable the  
30 selection of a glycosylated polylysine derivative which effects high efficiency transfection of a particular cell type.

Taking alveolar macrophages

of other genes into that particular cell type. One example of such a cell transfection kit is Perfect Lipid™.

Thus, according to the invention, a kit is provided which comprises a selection of glycosylated polylysine derivatives and optionally includes at least one type of reporter DNA molecule. The selection of polylysine derivatives includes polylysine substituted with lactose,  $\alpha$ -glucose,  $\beta$ -galactose, mannose, mannose-6-phosphate, fucose and N-acetylglucosamine. The amount of each polylysine derivative to be used for transfection of cells will vary depending on any number of factors including the type of DNA and type of cells to be transfected. Typically, the ratio of polylysine derivative to DNA will be from about 1:1 to about 1:15.

By the term "a selection of polylysine derivatives," as used herein, is meant a combination of polylysine derivatives, each one of which is packaged individually so that they are not mixed together.

Reporter DNAs which can be optionally included in the kit include, but are not limited to plasmids or other forms of DNA comprising genes which encode chloramphenicol acetyl transferase, luciferase, green fluorescent protein gene,  $\beta$ -galactosidase, and the like. Essentially, a reporter DNA includes any DNA encoding a product which is detectable in transfected cells. The reporter DNA also comprises a promoter/regulatory sequence for driving expression of the DNA in a cell in which the DNA is transfected. Such promoter/regulatory sequences include, but are not limited to, constitutive promoter /regulatory sequences, such as, but not limited to the SV40 early promoter, the cytomegalovirus immediate early promoter and the Rous sarcoma virus promoter/enhancer, tissue specific promoter/regulatory sequences and inducible promoter sequences. The type of reporter gene and the promoter sequence to which it is operably linked will depend on the type of cells to be transfected and will be readily apparent to one of skill in the art of cell transfection and gene expression. Typically, the concentration of DNA used in a transfection assay will be about 1  $\mu$ g to about 40  $\mu$ g of plasmid mixed in the desired ratio with the glycosylated polylysine.

Also optionally included in the kit are compounds which further enhance the transfection of glycosylated polylysine DNA complexes into cells.

In the case of cell transfection *in vitro*, the instructions comprise directions on how to mix the desired nucleic acid and polylysine to the appropriate proportions, how to treat cells prior to, during and following addition of the transfection mixture to the cells, and how to assess CFTR activity in CFTR  
5 transfected cells. These instructions simply embody the examples provided herein.

In the case of administration of isolated CFTR nucleic acid complexed with glycosylated polylysine to a human, the kit may also comprise a nebulizer into which the transfection mixture is placed. Thus, in this case, instructions for using the kit comprise directions for mixing the isolated nucleic  
10 acid and glycosylated polylysine, and directions including dosages, as described herein, for administering the complex to a human. Such administration directions may also include instructions as to the amount of transfection mixture to be added to the nebulizer and the manner in which the nebulizer is to be used on the patient. Such instructions and directions will depend on factors such as the age of the  
15 individual and the severity of the disease, but in any event, will be apparent to the artisan skilled in the treatment of CF.

The invention should not be construed to be limited to transfection of cells *in vivo* in an animal for the purpose of treatment of a disease. Rather, the invention should also be construed to include transfection of a variety of cells *in vitro* using glycosylated polylysine as a delivery vehicle. Currently available  
20 technology for delivery of DNA to cells *in vitro* is limited with respect to the efficiency with which the DNA is delivered. This is particularly true in the case of cells which are considered to be difficult to transfect, such as monocytes and macrophages, for example. Several commercially available cell transfection kits  
25 include a range of lipid compositions which can be mixed with a reporter DNA for transfection into a variety of cell types. The reporter DNA typically comprises a gene encoding a detectable protein product operably linked to a promoter/enhancer sequence for driving expression of the reporter gene when transfected into cells. The lipid composition which effects the most efficient transfection of reporter DNA  
30 into a given cell type is then selected as the composition of choice for transfection

accomplished by following the procedure described in Sekhon *et al.* (1995, *Nature Med.* 1:1201-1203).

Pharmaceutical compositions suitable for administration of glycosylated polylysine nucleic acid complexes to the airway epithelial cells of an animal *in vivo* include, but are not limited to, any of the compositions described  
5 herein for transfection of cells in culture.

Also encompassed by the invention is a method of identifying a compound capable of modulating the activity of CFTR. For example, cells in culture which are transfected with the CFTR gene may be used to identify a  
10 compound which has an effect on CFTR activity. Thus, cells which are transfected with the CFTR gene provide an *in vitro* system for the identification of compounds which modulate CFTR activity. To practice this aspect of the invention, transfected cells expressing CFTR may be treated with a compound which is predicted to affect CFTR activity, and the effect of the compound on CFTR activity may be assessed  
15 by using any of the procedures described herein. In this manner, compounds having an effect on CFTR activity are identified and can be further tested for their capability as therapeutic agents for treatment of CF, also as described herein.

The manner in which a compound capable of modulating CFTR activity is identified is straightforward and simple to practice one armed with the  
20 teaching described herein. For this reason, the invention should be construed to include any and all compounds which are identified following the methods described herein.

Also included in the invention is a kit comprising an isolated nucleic acid comprising a DNA useful for treating a human, for example, but not limited to  
25 CFTR, or a biologically active fragment thereof, a glycosylated polylysine and instructions for using the kit. Optionally, the kit may include one or more of glycerol, fusogenic peptide or chloroquine. The kit is useful for transfection of airway epithelial cells, useful, for example, for the identification of compounds capable of modulating CFTR activity, and is also useful for treatment of humans  
30 having CF. The instructions for using the kit therefore depend on the procedure for which the kit is to be used.

non-human primates (Simon *et al.*, 1993, *Human Gene Therapy* 4:771-780).

Transgenic animal models may also be useful in the invention.

According to the methods of the invention, glycosylated polylysine complexed to CFTR DNA may be administered to a human having CF in a manner similar to that described for the animal models discussed herein. Essentially, the human is anesthetized unless the DNA is to be administered via an aerosol nebulizer, and the DNA/polylysine complex is administered by bronchoscopy, or by using a tracheal catheter, at doses of about 500 µg to about 10 mg of DNA and an appropriate amount of glycosylated polylysine in a volume of about 1 ml to about 100 ml depending on the age and size of the individual and the severity of the disease. Typically, a normal adult will receive about 50 ml of a DNA solution having about 5 mg of CFTR DNA. The administration of a compound to a human by aerosol, bronchoscopy, or tracheal catheter, is well known in the art and is described, for example, in Curiel *et al.* (1996, *Am. J. Respir. Cell Mol. Biol.* 14:1-18). It will be appreciated that the precise method of administration of nucleic acid complexed with glycosylated polylysine to a human will depend on any number of factors including the age of the individual and the severity of the disease. The precise mode of treatment of a human will be apparent to the artisan skilled in the treatment of CF and will be tailored by the artisan to the individual being treated.

The DNA/polylysine complex is administered to the human about once a month or less, or about once every two months, or even about once every three months. The treatment regime to be used will depend on several factors including the age of the individual, the extent of the CF symptoms and the overall health of the individual, the length of time since the onset of symptoms, etc.

The invention should also be construed to include treatment of disease *in utero* using glycosylated polylysine DNA complexes. Again, using CFTR as an example, given the availability of genetic tests capable of identifying a defective CFTR gene, it is now possible to determine whether a fetus *in utero* has a defective CFTR gene. Such defects can be corrected *in utero*, prior to the onset of symptoms following birth, thereby preventing many of the sequelae of CF experienced by children and young adults with this disease. Treatment *in utero* is



*situ* hybridization and, in addition, expression is measured in any CFTR assay such as the assays described herein in the experimental details section.

For transfection of cells *in vivo*, a plasmid or other DNA molecule encoding the desired protein is complexed with glycosylated polylysine and the complex is administered to an animal following the procedures described herein for transfer of reporter plasmids to animal airway epithelial cells. Expression of the protein is assessed as described herein. Administration of lactose substituted polylysine/DNA complex to airway epithelial cells of animals may be accomplished by aerosol through the nasal passages, by bronchoscopy or by any other method available in the art, such as by tracheal catheter.

Using CFTR as an example, but appreciating that the invention is not limited solely to the use of CFTR, procedures for transfection of cells *in vivo* are now described. In addition to mice, rabbits and other vertebrate animals may be used, including non-human primate animals, to examine the introduction of DNA, for example, CFTR into the airway epithelial cells of these animals using the methods described herein.

To test the effectiveness of, for example, CFTR added to airway epithelial cells *in vivo*, the tracheal xenograft model may be used (Engelhard *et al.*, 1992, *J. Clin. Invest.* **90**:2598-2607). This model is described in detail herein in the experimental details section. Essentially, the model comprises an immunodeficient mouse having a denuded rat-trachea transplant positioned under the skin on which CF airway epithelial cells are grafted. CFTR nucleic acid complex with glycosylated polylysine suspended in a suitable transfection mixture is administered to the animals at the site of the xenograft. After a period of time, generally about four hours, the mixture is removed. Correction of the CF-associated defect is then assessed by measuring transepithelial potential difference with amiloride stimulation after about thirty six to about forty hours post transfection.

Alternatively, other animals may also be used for administration of CFTR using glycosylated polylysine. Such animal models include, but are not limited to, adult mice and rabbits (Lisby *et al.*, 1996, *Pediatr. Res.* **39**: 389A) and

The invention should thus be construed to include nucleic acid encoding desired proteins and fragments of nucleic acid encoding desired polypeptides; and, nucleic acids and fragments of nucleic acids which are in the antisense orientation to nucleic acid encoding the desired protein or polypeptide.

5 By the term "transfection" as used herein, is meant the transport of nucleic acid into a cell and the expression of the nucleic acid therein.

The term "expression of a nucleic acid " as used herein means the synthesis of the protein product encoded by the nucleic acid.

A suitable transfection mixture for transfer of nucleic acid into  
10 airway epithelial cells comprises any ordinary transfection mixture available in the art, including, but not limited to, isotonic medium, for example, DMEM, which is preferably serum-free. In addition, other compounds may be added to the transfection mixture for the purpose of improving the stability of the complex and/or improving the transfection efficiency in the desired cells. Such compounds  
15 include, but are not limited to, chloroquine, glycerol and fusogenic peptides. It will be appreciated that the amount of such compounds to be added to the transfection mixture will vary depending on any number of factors, including, but not limited to, the type of compound being used, whether transfection is conducted *in vitro* or *in vivo*, the size of the nucleic acid to be transfected and the amount and relative ratios  
20 of the nucleic acid to substituted polylysine. Examples of the use of such enhancing compounds are included herein in the experimental details section.

For transfection *in vitro*, the nucleic acid encoding the gene to be expressed and glycosylated polylysine are added to cells in the appropriate transfection mixture. The cells are incubated for a period of time, generally about  
25 three to about four hours, and the cells are then washed, growth medium is added and the cells are incubated for an additional about twenty four to about seventy two hours. Expression of the gene is assessed using any number of detection methods for the production of protein, including, but not limited to immunological protein detection methods, such as Western blotting, immunofluorescence, ELISA, and the  
30 like. When the nucleic acid to be transfected is CFTR DNA, expression of CFTR nucleic acid is assessed using any of the aforementioned techniques including *in*

capable of base pairing with each other, then the nucleic acids are considered to be complementary to each other at this position. Thus, two nucleic acids are complementary to each other when a substantial number (at least 50%) of corresponding positions in each of the molecules are occupied by nucleotides which normally base pair with each other (e.g., A:T and G:C nucleotide pairs). As defined  
5 herein, an antisense sequence is complementary to the sequence of a double stranded DNA molecule encoding a protein. It is not necessary that the antisense sequence be complementary solely to the coding portion of the coding strand of the DNA molecule. The antisense sequence may be complementary to regulatory  
10 sequences specified on the coding strand of a DNA molecule encoding a protein, which regulatory sequences control expression of the coding sequences.

The antisense nucleic acids which are useful in the present invention include those which have been modified to enhance their stability or otherwise enhance their ability to inhibit gene expression. Antisense nucleic acids which  
15 contain at least one phosphorothioate modification are known to confer upon the oligonucleotide enhanced resistance to nucleases. Specific examples of modified oligonucleotides include those which contain phosphorothioate, phosphotriester, methyl phosphonate, short chain alkyl or cycloalkyl intersugar linkages, or short chain heteroatomic or heterocyclic intersugar ("backbone") linkages. In addition,  
20 oligonucleotides having morpholino backbone structures (U.S. Patent No: 5,034,506) or polyamide backbone structures (Nielsen et al., 1991, Science 254: 1497) may also be used. The examples of antisense oligonucleotide modifications described herein are not exhaustive and it is understood that the invention includes additional modifications of the oligonucleotides of the invention which  
25 modifications serve to enhance the therapeutic properties of the oligonucleotide without appreciable alteration of the basic sequence of the oligonucleotide.

Methods of preparing the oligonucleotides used in accordance with this invention are routine in the art, for example, solid phase synthesis is a well known technique commonly used to synthesize such oligonucleotides. It is also  
30 well known to use similar techniques to prepare other oligonucleotides such as phosphorothioate and alkylated derivatives.

halogens in cells, wherein an increase in efflux of the halogen in cells treated with the putative CFTR gene fragment, which cells are also stimulated by either forskolin or cyclic AMP, identifies the fragment as a biologically active fragment of the CFTR gene.

5 Also contemplated in the invention is the transfection of cells either *in vivo* or *in vitro* with a DNA molecule which is in the antisense orientation with respect to the coding strand of double stranded DNA. Thus, the invention also includes an isolated nucleic acid having a nucleotide sequence which is in the antisense orientation (*i.e.*, is complementary) to a portion or all of a nucleic acid  
10 encoding a gene, the expression of which is detrimental to a host or cell.

During the development of certain respiratory diseases in a human, genes are expressed, which if inhibited, would serve to arrest the development of the disease. Such genes are referred to herein as genes which are required for the development of the respiratory disease. For example, during the development of  
15 asthma in a human, cytokines such as IL-4 and IL-5, and leukotrienes are expressed which facilitate the development of an asthma attack. Inhibition of expression of the genes encoding these products would serve to arrest the development of the asthma attack. The invention therefore contemplates the use of DNA molecules which are in the antisense orientation to genes encoding products which are  
20 required for the development of a respiratory disease.

By "complementary" to all or a portion of a gene, as used herein, is meant a sequence of nucleic acid which does not encode the protein specified by the gene. Rather, the sequence which is being expressed in the cells is identical to the non-coding strand of the subject gene and thus, does not encode the protein.

25 The terms "complementary" and "antisense" as used herein, are not entirely synonymous. "Antisense" refers particularly to the nucleic acid sequence of the non-coding strand of a double stranded DNA molecule encoding a protein, or to a sequence which is substantially homologous to the non-coding strand. "Complementary" as used herein refers to the broad concept of subunit sequence  
30 complementarity between two nucleic acids, e.g., two DNA molecules. When a nucleotide position in both of the molecules is occupied by nucleotides normally

By the term "substantially homologous" as used herein, is meant DNA or RNA which is about 50% homologous, more preferably about 70% homologous, even more preferably about 80% homologous and most preferably about 90% homologous to the desired nucleic acid. Genes which are homologous to the desired gene should be construed to be included in the invention provided they encode a protein or polypeptide having a biological activity substantially similar to that of the desired gene.

The desired nucleic acid useful in the invention is nucleic acid encoding a gene which when administered to a mammal, preferably, a human, serves to alleviate the symptoms of or serves to cure a respiratory disease in the mammal. The preferred diseases to be treated include cystic fibrosis, asthma, emphysema, idiopathic pulmonary fibrosis and congenital surfactant deficiency. The preferred DNAs to be used therefore include the *CFTR* gene, an asthma gene, the  $\alpha 1$ AT gene, a gene affecting idiopathic pulmonary fibrosis and the SP-B and SB-C genes. The invention should also be construed to include biologically active fragments of each of these genes and should further be construed to include all forms of DNA, including cDNA, genomic DNA and synthetic DNA.

By the term "biologically active," as used herein, is meant a fragment of DNA which encodes a polypeptide that retains the biological activity of the full length protein from which the polypeptide is derived.

Using CFTR as an example, but understanding that the invention is not limited thereto, biologically active fragments of an isolated nucleic acid encoding CFTR will ordinarily be at least about 240 contiguous nucleic acids in length, typically at least about 500 contiguous nucleic acids, more typically at least about 1000 continuous nucleic acids, and even more typically, at least about 4000 to about 6000 contiguous nucleic acids in length. A fragment of nucleic acid encoding CFTR must be biologically active in order to be useful in the methods of the invention. The biological activity of CFTR is defined as an increase in efflux of chloride ions in epithelial cells following stimulation of the cells with either forskolin or cyclic AMP. In addition to the measurement of chloride ion efflux, CFTR activity may also be identified by measuring iodine efflux or efflux of other

encoding additional polypeptide sequence and optionally having promoter/regulatory sequences fused thereto to enhance or control expression of a protein encoded thereby.

5 The isolated nucleic acid should be construed to include a DNA or RNA sequence specifying the desired DNA or RNA, and any modified forms thereof, including modifications of the DNA or RNA which render the nucleotide sequence more stable when it is cell free or when it is associated with a cell. Modifications of nucleic acids may also be used to enhance the efficiency with which a nucleic sequence is taken up by a cell or the efficiency with which it is expressed in a cell. Any and all combinations of modifications of the nucleotide sequences are contemplated in the present invention.

The invention should not be construed as being limited solely to DNA or RNA encoding the aforementioned genes. Once armed with the present invention, it is readily apparent to one skilled in the art that DNA or RNA molecules which are homologous to the aforementioned genes and which encode proteins or peptides which are substantially similar in function to the function of the proteins encoded by the aforementioned genes may be obtained by following the well known procedures described in the art for the isolation of DNA or RNA molecules which are homologous to known DNA or RNA molecules.

20 "Homologous" as used herein, refers to the subunit sequence similarity between two polymeric molecules, *e.g.*, between two nucleic acid molecules, *e.g.*, two DNA molecules or two RNA molecules, or between two polypeptide molecules. When a subunit position in both of the two molecules is occupied by the same monomeric subunit, *e.g.*, if a position in each of two DNA molecules is occupied by adenine, then they are homologous at that position. The homology between two sequences is a direct function of the number of matching or homologous positions, *e.g.*, if half (*e.g.*, five positions in a polymer ten subunits in length) of the positions in two compound sequences are homologous then the two sequences are 50% homologous, if 90% of the positions, *e.g.*, 9 of 10, are matched or homologous, the two sequences share 90% homology. By way of example, the DNA sequences 3'ATTGCC5' and 3'TATGCG5' share 50% homology.

for expression of the product therefrom. In addition, the DNA molecule may include an origin of DNA replication which confers on the DNA the ability to replicate in the cell in which it has been introduced. Such origins of DNA replication are preferably eukaryotic replication origins and include any origin of DNA replication which facilitate replication of the DNA in a cell. Eukaryotic origins of DNA replication include, but are not limited to, the Epstein Barr virus DNA replication origin and associated DNA elements for facilitating replication, and the SV40 origin of replication. The ligation of an origin of DNA replication to a desired DNA molecule is well known in the art and is described, for example, in Sambrook et al. (1989, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor, NY). DNA which comprises an origin of DNA replication is useful in that it may have a longer therapeutic effect in an animal to which it is administered than DNA which does not comprise such an origin.

The DNA molecule to be transfected into cells, whether or not it is accompanied by additional sequences, is referred to herein as "an isolated nucleic acid molecule." It will be appreciated that as technology for the isolation of and modification of RNA advances, it may be possible to use an RNA molecule in place of the DNA molecule described herein. Thus, the term "isolated nucleic acid" should be construed to encompass both DNA and RNA.

By "isolated nucleic acid" as used herein is meant a nucleic acid sequence, a DNA or and RNA sequence, which has been separated from the sequences which flank it in a naturally occurring state, *e.g.*, a DNA or RNA fragment which has been removed from the sequences which are normally adjacent to the fragment, *e.g.*, the sequences adjacent to the fragment in a genome in which it naturally occurs. The term also applies to nucleic acids which have been substantially purified from other components which naturally accompany the nucleic acid (*e.g.*, RNA, DNA or protein) in its natural state. The term therefore includes, for example, a recombinant DNA which is incorporated into a vector or into an autonomously replicating plasmid, or as a cDNA or a genomic or cDNA fragment produced by PCR or restriction enzyme digestion, independent of other sequences. It also includes a recombinant DNA which is part of a hybrid gene

histamine, and the like. Thus, any gene having the desired effect of alleviating asthma is included in the definition of an asthma gene as used herein.

When the disease to be treated is idiopathic pulmonary fibrosis, a DNA molecule, or a biologically active fragment thereof, comprising a gene whose protein product serves to alleviate idiopathic pulmonary fibrosis will be used.

By the term "a gene affecting idiopathic pulmonary fibrosis" as used herein, is meant a gene which is involved in the events leading to the onset or maintenance of this disease. Similar to the situation with respect to asthma, any gene having the desired effect of alleviating idiopathic pulmonary fibrosis is included in the definition of a gene affecting idiopathic pulmonary fibrosis as used herein.

In the case of emphysema, DNA comprising  $\alpha$ 1AT, or a biologically active fragment thereof, will be used to transfect cells and, in the case of congenital deficiency of surfactant protein, DNA comprising SP-B and/or SP-C will be used.

The various sources of known DNA's which may be used are either described or are referenced herein. For example, a source of CFTR DNA is described herein in the experimental examples section. A source of  $\alpha$ 1AT DNA is referenced in Canonico (*supra*) and a source of SP-B and SP-C DNAs is referenced in Whitsett et al. (1995, Physiological Reviews 75:749-757) and Noguee et al. (1994, J. Clin. Invest. 93:1860-1863). The source of other DNA's which may be used will be apparent to those of skill in the art of respiratory disease.

It will be appreciated that when the cells to be transfected are cells in culture, whether they are macrophages, tumor cells, fibroblast cell cultures or any other cell type, the DNA which will be used to transfect the cells will depend upon the particular application desired.

The DNA molecule may be contained within a plasmid, or may simply comprise the sequences to be transfected and any additional nucleic acid sequences which render the DNA more stable, or which enhance expression of the desired gene. Thus, the DNA molecule may include other sequences which enhance the expression of the DNA in a cell in which it has been introduced. For example, the DNA molecule may include promoter sequences, as described herein,



glycosyl residues results in high efficiency transfection of cells. Preferably, substitution of about 12% to about 40% of the amino groups of the polylysine molecule with glycosyl residues is optimal for transfection of cells.

It has been discovered in the present invention that the efficiency by which airway epithelial cells are transfected with nucleic acid complexed with substituted polylysine is affected by the weight to weight ratio of polylysine to DNA. Thus, in the case of polylysine substituted with lactose, preferably, the weight to weight ratio of lactosylated polylysine to DNA ranges from about one to one to about fifteen to one. More preferably, the weight to weight ratio of lactosylated polylysine to DNA ranges from about two to one to about nine to one. Even more preferably, the weight to weight ratio of lactosylated polylysine to DNA ranges from about three to one to about nine to one. When polylysine is substituted with sugars other than lactose, it will be appreciated that the weight to weight ratio of substituted polylysine to DNA will vary depending upon the type of sugar and the type and/or the size of the DNA being used. It is anticipated that the weight to weight ratio of substituted polylysine to DNA will be generally within the ranges given for lactosylated polylysine; however, the invention should not be construed as being limited to these ratios when glycosyl sugars other than lactose are used.

When transfection of cells *in vivo* in a mammal is contemplated, the type of DNA to be transfected will depend upon the disease to be treated. For example, for treatment of CF, a DNA molecule comprising the *CFTR* gene, or a biologically active fragment thereof, will be used. When the disease to be treated is asthma, a DNA molecule, or a biologically active fragment thereof, comprising a gene whose protein product serves to alleviate asthma will be used.

By the term "an asthma gene" as used herein, is meant a gene whose protein product has the effect of alleviating asthma. Such genes include those involved in the cascade of events leading to an asthma attack, as well as known or heretofore unknown genes which may be involved in the susceptibility of some individuals to asthma. Genes which are involved in the asthma cascade include genes encoding cytokines, such as, but not limited to, IL-4 and IL-5, genes affecting leukotriene synthesis, genes encoding proteins which govern the production of

of substituted polylysine derivatives is provided herein in the experimental details section.

To form a substituted polylysine DNA complex, the substituted polylysine derivative is added to DNA in a controlled manner in order that precipitation of the complex so formed does not occur. Generally, an amount of substituted polylysine is added to DNA in solution at a rate of several microliters per about 30 to about 60 seconds. Although, the examples provided in the experimental details section herein recite the rate of addition of substituted polylysine to DNA as being 15  $\mu$ l per 30 to 60 seconds, it will be appreciated that this rate may vary depending upon the precise amounts and types of substituted polylysine and DNA being mixed.

The types of substituted polylysine which are suitable for transfection of cells will vary depending on the cells to be used. When the polylysine DNA complex is to be used for transfection of airway epithelial cells *in vivo* in a mammal, preferably a human, then lactosylated polylysine is the polylysine derivative of choice. In contrast, in the case of immortalized airway epithelial cells, lactose,  $\alpha$ -glucose,  $\beta$ -galactose, mannose, mannose-6-phosphate, fucose or N-acetylglucosamine substituted polylysine may be used. Similarly, as will be described herein in greater detail, when cells which are not airway epithelial cells are to be transfected, glycosyl residues other than lactose may be used.

By the use of the term "glycosylated polylysine" as used herein, is meant a polylysine molecule which has substituted thereon glycosyl moieties. Thus, the term "glycosylated polylysine" may be distinguished from the term "gluconoylated polylysine" since a gluconoylated polylysine molecule has substituted thereon gluconoyl moieties. Glycosyl moieties differ from gluconoyl moieties in that glycosyl moieties comprise sugar, *i.e.*, carbohydrate molecules, whereas gluconoyl moieties are not considered in the art to be carbohydrate molecules since the characteristic ring structure is open. There are no gluconoyl moieties on the glycosylated polylysine molecules of the present invention.

It has been discovered in the present invention that substitution of about 10% to about 60% of the amino groups of the polylysine molecule with

were previously transfected with wild type CFTR and were subsequently transfected with pCMV*Luc*/lactosylated polylysine at a DNA to lactosylated polylysine ratio of 1:3 and the indicated concentrations of chloroquine with or without 5% glycerol.

5

## DETAILED DESCRIPTION OF THE INVENTION

It has been discovered that substituted polylysines are capable of facilitating gene delivery to airway epithelial cells thereby providing an alternative to viral vectors for the delivery of genes into airway epithelial cells in patients having respiratory disease. Until the present invention, it was not known that airway epithelial cells could be transfected with DNA complexed to substituted polylysine. The substituted polylysines useful in the present invention comprise polylysine which is partially neutralized by blocking a number of positive-charged residues with sugar groups.

It has been discovered that polylysine substituted with  $\alpha$ -glucose,  $\beta$ -galactose or lactose are equally efficient as DNA transfer vehicles for the introduction of DNA into immortalized airway epithelial cells. However, polylysine substituted with lactose is superior to other substituted polylysines for effecting transfer of DNA into primary airway epithelial cells.

Thus, the invention includes a substituted polylysine which comprises either mono or disaccharide residues on a specified number of amino groups.

The use of polylysine substituted with mono or disaccharides for gene delivery is superior compared with other polylysine derivatives and compared with viral vectors in that mono or disaccharide substituted polylysine is non-immunogenic (Levine, 1964, *Proc. Soc. Exp. Biol. Med.* **116**:1127-1131; Fiume *et al.*, 1994, *Biochem. Pharmacol.* **47**:643-650).

The generation of substituted polylysine derivatives is well known in the art and is described, for example, in Midoux *et al.* (*supra*) and in Martinez-Fong *et al.*, 1994, *Hepatology*, **20**:1602-1608). A detailed description of the generation

30

(1994, *Biochim. Biophys. Acta* **1195**:96-102). Figure 15A (Panel A) comprises data obtained from T-84 tumor cells which are the prototype of CFTR-containing cells. Figure 15B (Panel B) comprises data obtained from CFT1 cells whose defect has been corrected by the addition of wild type CFTR. CF/T43 cells exhibited no response when similarly treated.

**Figure 16** is a series of images of photomicrographs demonstrating high efficiency expression of  $\beta$ -galactosidase following transfection with pCMVLacZ. The efficiency of gene transfer into CF/T43 cells was examined using 60  $\mu$ g of lactosylated polylysine complexed to 20  $\mu$ g of pCMVLacZ in the presence of 100  $\mu$ M chloroquine and 5% glycerol. The cells were transfected for four hours at 37°C either once or three times on three sequential days, followed by incubation in growth medium. Expression of LacZ was detected following fixation in 2% paraformaldehyde/0.2% glutaraldehyde followed by incubation for 18 hours at 37°C in X-gal stain. The cells were subsequently examined in a Nikon Diaphot 300 microscope (12.5 X magnification). Figure 16A is an image of cells transfected with pCMVLacZ coupled to lactosylated polylysine, wherein the cells were transfected for three consecutive days. Figure 16B is an image of cells transfected as in Figure 16A, wherein the cells were transfected once. Figure 16C is an image of cells transfected as in Figure 16A, wherein the cells were transfected with the plasmid but without lactosylated polylysine.

**Figure 17** is a series of images of photomicrographs of cells depicting expression of CFTR in CF airway epithelial cells in primary culture. Expression of CFTR was detected by *in situ* hybridization. Figure 17A depicts primary cells grown on coverslips which were transfected with pAdCFTR complexed with lactosylated polylysine. Figure 17B depicts primary cells grown on coverslips which were transfected with pAdCFTR which was not complexed to lactosylated polylysine.

**Figure 18** is a series of graphs depicting the effect of potentiating agents on human airway epithelial cell lines. Figure 18A: BEAS2B, immortalized nonCF airway epithelial cells.; Figure 18B: CF/T1 (wild type) immortalized airway epithelial cells obtained from a CF patient having the  $\Delta$ F508 mutation. These cells

**Figure 10** is a graph depicting enhancement of reporter gene expression in CF/T43 cells transfected with a plasmid encoding the reporter gene, pCMVLuc, complexed to lactosylated polylysine, wherein the transfection was conducted in the presence (open circles) or absence (closed circles) of 100  $\mu$ M chloroquine and the indicated concentrations of glycerol.

**Figure 11** is a graph depicting enhancement of reporter gene expression in primary airway epithelial cells transfected with a plasmid encoding the reporter gene, pCMVLuc, complexed to lactosylated polylysine, wherein the transfection was conducted in the presence of the indicated concentrations of glycerol without the addition of chloroquine. A transfection assay conducted in the presence of 50  $\mu$ M chloroquine was used for comparison.

**Figure 12** is a graph depicting enhancement of reporter gene expression in CF/T43 cells transfected with a plasmid encoding the reporter gene, pCMVLuc, complexed to gluconoylated polylysine, in the presence of the indicated concentrations of fusogenic peptide or chloroquine.

**Figure 13** is a graph depicting the enhancement of reporter gene expression in CF/T43 cells transfected with a plasmid encoding the reporter gene pCMVLuc coupled to lactosylated polylysine in the presence of the indicated concentrations of different additives as noted.

**Figure 14** is a graph depicting the enhancement of reporter gene expression in CF/T43 cells transfected with pCMVLuc coupled to different concentrations of several preparations of lactosylated polylysines. PM 186 is presented as the mean value  $1.08 \pm 0.02$  RLU ( $\times 10^8$ ;  $n = 10$ ).

**Figure 15** is a series of graphs depicting assays for Cl<sup>-</sup> efflux using <sup>125</sup>I. A concentration of  $2 \times 10^5$  cells were cultured in 15 mm wells for three to four days. The cells were washed and <sup>125</sup>I was added in efflux medium, following which, 1 ml fractions were obtained from the cultures. A mixture comprising 20 mM forskolin, 250 mM cAMP and 500 mM isobutyl-1-methylxanthine. (IBMX) was added to the cultures at the time indicated by the arrow, and removal of 1 ml fractions from the cultures at the indicated times was continued. Efflux was measured and was expressed as the rate per minute as described in Santos et al.

polylysines complexed with pCMV*Luc* and the level of gene expression in these cells was compared with that in cells transfected with gluconoylated polylysine complexed with pCMV*Luc*. Following transfection in the presence of 100  $\mu$ M chloroquine, the cells were incubated in LHC-9 medium (Biofluids Inc.) and the amount of luciferase activity expressed in the cells was measured. The results in **Figure 6A** and **Figure 6B** are those obtained from two separately conducted experiments in two different primary cultures of cells.

**Figure 7**, comprising parts A and B, is a series of graphs depicting reporter gene expression in non-CF tracheal cells in primary culture using glycosylated polylysines as the vehicle. Cells were transfected with glycosylated polylysines complexed with pCMV*Luc* and the level of gene expression in these cells was compared with that in cells transfected with gluconoylated polylysine complexed with pCMV*Luc*. Following transfection in the presence of 100  $\mu$ M chloroquine, the cells were incubated in LHC-9 medium and the amount of luciferase activity in the cells was measured. The results in **Figure 7A** and **Figure 7B** are those obtained from two separately conducted experiments in two different primary cultures of cells.

**Figure 8** is a graph depicting the effect of chloroquine on reporter gene expression in non-CF tracheal cells in primary culture. Cells were transfected for 4 hours with lactosylated polylysine and pCMV*Luc* at a ratio of 3:1 and the indicated concentrations of chloroquine. Following transfection, cells were incubated for 48 hours and the amount of luciferase activity in the cells was subsequently measured.

**Figure 9** is a series of photomicrographs depicting binding of Lac-BSA-FITC to CF/T43 cells. CF/T43 cells were grown on coverslips for 24 hours, the culture medium was removed and the cells were processed for the binding assay. Lac-BSA-FITC (100  $\mu$ g per ml) was added to the cells for 30 minutes at 4°C in the absence of (**Figure 9A**), and the presence of (**Figure 9B**) 0.1 M lactose. The cells were examined in a Nikon Diaphot 300 microscope (Magnification is X 250).

The amount of luciferase expressed in the cells was then measured. When cells were transfected for 2 hours,  $8.5 \times 10^6$  RLU per mg of protein was detected.

In **Figure 3B**, CF/T43 cells were transfected for 4 hours with the plasmid pCMV*Luc* and gluconoylated polylysine. Following transfection, the cells were washed and were incubated in KGM medium for the times indicated on the figure. At those indicated times, the amount of luciferase expressed in the cells was assessed. At 6 and 12 hours following the 4 hour transfection period,  $3 \times 10^4$  and  $1.3 \times 10^5$  RLU, respectively, were detected per well.

**Figure 4** is a graph depicting reporter gene expression in cells transfected with various glycosylated polylysines as vehicles. The substituted polylysines indicated on the figure were complexed with  $5 \mu\text{g}$  of the reporter plasmid pCMV*Luc*. CF/T43 cells were transfected for 4 hours, washed and incubated in KGM medium and luciferase activity was measured. "None" indicates that non substituted polylysine was added to the transfection mixture; "GlcA" is gluconoylated polylysine.

**Figure 5**, comprising parts A, B and C, is a series of graphs depicting optimization of expression of a reporter gene in transfected CF/T43 cells using lactosylated polylysine as a vehicle.

In **Figure 5A**, one  $\mu\text{g}$  of pCMV*Luc* was used in the presence of 100  $\mu\text{M}$  chloroquine while the concentration of lactosylated polylysine was varied as indicated.

In **Figure 5B**, the time of the actual transfection was varied from 2 to 10 hours using  $3 \mu\text{g}$  lactosylated polylysine and 1  $\mu\text{g}$  of reporter gene pUT 650 (*i.e.*, in a w/w ratio of 3:1) in the presence of 100  $\mu\text{M}$  chloroquine.

**Figure 5C**. Chloroquine at the indicated concentrations was added to the transfection medium containing lactosylated polylysine/pUT 650 complex at a ratio of 3:1 for 4 hours. Cells were washed, incubated in KGM medium for 48 hours, and luciferase activity was measured. In the absence of chloroquine,  $2.5 \times 10^6$  RLU per mg of protein was expressed in the cells.

**Figure 6**, comprising parts A and B, is a series of graphs depicting reporter gene expression in CF airway epithelial cells in primary culture using glycosylated polylysines as the vehicle. Cells were transfected with glycosylated

## BRIEF DESCRIPTION OF THE DRAWINGS

**Figure 1** is a graph depicting the influence of chloroquine on gene expression. Chloroquine at the indicated concentrations, was added to transfection medium containing gluconoylated polylysine and the plasmid pUT 650. The mixture was added to CF/T43 cells which were then incubated for 4 hours. Following subsequent culture of the cells for 48 hours in KGM medium (Clonetics Corp.), the cells were lysed and the level of expression of luciferase was assessed using a luminometer. The relative light units (RLU) were measured at 5 s and the amount of RLU per mg of protein was calculated. When cells were incubated in the absence of chloroquine,  $6.8 \times 10^5$  RLU per mg of protein was detected.

**Figure 2**, comprising parts A and B, is a series of graphs depicting the relationship between the concentration of plasmid and cell number and transfected gene expression.

In **Figure 2A**, a transfection assay was performed as described in Figure 1 wherein the amount of gluconoylated polylysine:pUT 650 (2:1, weight/weight) was varied as indicated, in relationship to the cell number which was maintained at a constant value of  $1.5 \times 10^5$  cells per culture well. When no plasmid was present in the mixture, a blank value was obtained.

In **Figure 2B**, a transfection assay was performed as described in Figure 1, wherein a constant amount (1  $\mu$ g) of the plasmid, pCMV*Luc* and gluconoylated polylysine (2  $\mu$ g) was added to a varying number of CF/T43 cells per well as indicated in the figure.

**Figure 3**, comprising parts A and B, is a series of graphs depicting the effect of transfection time on luciferase gene expression (A) and the effect of incubation time post transfection on luciferase gene expression (B) following transfection of CF/T43 cells using gluconoylated polylysine as a vehicle.

In **Figure 3A**, the level of expression of the reporter gene, pUT 650, was assessed at various times post-transfection of CF/T43 cells. Chloroquine (100  $\mu$ M) was included in the transfection mixture. The DNA complex was added to CF/T43 cells in the transfection medium and at various times the transfection mixture was removed and the cells were incubated in KGM medium for 48 hours.



The kit may further comprise a reporter DNA and may also further comprise at least one of chloroquine, glycerol and a fusogenic peptide.

Preferably, the glycosylated polylysine in the kit has a sugar component selected from the group consisting of lactose,  $\alpha$ -glucose,  $\beta$ -galactose, mannose, mannose-6-phosphate, fucose and N-acetylglucosamine.

Also preferably, the reporter DNA is selected from the group consisting of a chloramphenicol acetyl transferase gene, a luciferase gene, a green fluorescent protein gene, and a  $\beta$ -galactosidase gene.

The invention also includes a nebulizer having a composition comprising a complex comprising an isolated nucleic acid and a glycosylated polylysine placed therein. Preferably, the isolated nucleic acid is DNA encoding CFTR and the glycosylated polylysine is lactosylated polylysine.

In addition, the invention includes a bronchoscope having a composition comprising a complex comprising an isolated nucleic acid and a glycosylated polylysine placed therein. Preferably, the isolated nucleic acid is DNA encoding CFTR and the glycosylated polylysine is lactosylated polylysine.

There is further included in the invention an airway epithelial cell transfected with a complex comprising an isolated nucleic acid and a glycosylated polylysine. Preferably, the isolated nucleic acid is DNA encoding CFTR and the glycosylated polylysine is lactosylated polylysine.

In addition, the invention includes a composition for transfection of airway epithelial cells comprising a complex comprising an isolated nucleic acid and a lactosylated polylysine, wherein the isolated nucleic acid is DNA selected from the group consisting of DNA encoding CFTR, an asthma gene, DNA encoding  $\alpha$ 1AT, a gene affecting idiopathic pulmonary fibrosis, DNA encoding SP-B and DNA encoding SP-C. The composition may further at least one of chloroquine, glycerol and a fusogenic peptide.

In a preferred embodiment, the DNA is DNA encoding CFTR.

Preferably, the airway epithelial cells are transfected *in vitro* or are transfected *in vivo*.

acid encoding CFTR, or a biologically active fragment thereof, and a glycosylated polylysine. The composition may further comprise at least one of chloroquine, glycerol and a fusogenic peptide.

5 In a preferred embodiment of this aspect of the invention, the pharmaceutical composition is administered to the human by a means selected from the group consisting of aerosol nebulizer, bronchoscopy and injection *in utero*.

In yet another preferred embodiment of this aspect of the invention, the isolated nucleic acid comprises DNA, preferably, cDNA.

10 In another aspect of this method of the invention, the glycosylated polylysine has a sugar component selected from the group consisting of lactose,  $\alpha$ -glucose,  $\beta$ -galactose, mannose, mannose-6-phosphate, fucose and N-acetylglucosamine. Preferably, the glycosylated polylysine comprises lactosylated polylysine. More preferably, about 10% to about 60% of the amino groups of the polylysine have a lactose molecule substituted thereon. Also more preferably, the  
15 weight to weight ratio of lactosylated polylysine to DNA in the complex is about one to one to about fifteen to one. Even more preferably, the weight to weight ratio of lactosylated polylysine to DNA in the complex is about three to one to about nine to one.

20 There is further included in the invention a method of identifying a test compound capable of modulating the activity of CFTR. The method comprises transfecting airway epithelial cells in the presence or absence of the test compound with a complex comprising an isolated nucleic acid encoding CFTR, or a biologically active fragment thereof, and a glycosylated polylysine, and measuring the activity of CFTR in the cells, wherein a higher or a lower level of CFTR activity  
25 in the presence of the test compound compared with CFTR activity in cells in the absence of the test compound is an indication that the test compound is capable of modulating the activity of CFTR.

The invention includes a compound identified according to the just described method.

30 In addition, the invention relates to an *in vitro* cell transfection kit comprising a selection of glycosylated polylysines and instructions for using the kit.

In addition, the glycosylated polylysine in the pharmaceutical composition may have a sugar component selected from the group consisting of lactose,  $\alpha$ -glucose,  $\beta$ -galactose, mannose, mannose-6-phosphate, fucose and N-acetylglucosamine. Preferably, the glycosylated polylysine comprises lactosylated polylysine. More preferably, about 10% to about 60% of the amino groups of the polylysine have a lactose molecule substituted thereon. Even more preferably, about 12% to about 40% of the amino groups of the polylysine have a lactose molecule substituted thereon.

In another preferred embodiment, the weight to weight ratio of lactosylated polylysine to DNA in the complex is about one to one to about fifteen to one.

The invention also includes a lactosylated polylysine nucleic acid complex comprising DNA encoding CFTR, or a biologically active fragment thereof, and lactosylated polylysine, wherein about 10% to about 60% of the amino groups of the polylysine have a lactose molecule substituted thereon, and the weight to weight ratio of lactosylated polylysine to the DNA in the complex is about one to one to about fifteen to one, the complex being capable of transfecting airway epithelial cells when added thereto. Preferably, the weight to weight ratio of lactosylated polylysine to the isolated nucleic acid in the complex is about nine to one.

Also included in the invention is a kit comprising an isolated nucleic acid encoding CFTR, or a biologically active fragment thereof, a glycosylated polylysine and instructions for using the kit for transfection of airway epithelial cells.

In addition, the invention includes a kit comprising an isolated nucleic acid encoding CFTR, or a biologically active fragment thereof, a glycosylated polylysine and instructions for using the kit for treatment of cystic fibrosis in a human patient.

The invention further relates to a method of treating a human patient having cystic fibrosis, the method comprising administering to the human a pharmaceutical composition comprising a complex comprising an isolated nucleic

The pharmaceutical composition may further comprise at least one of chloroquine, glycerol and a fusogenic peptide.

In one embodiment, the respiratory disease is selected from the group consisting of cystic fibrosis, asthma, emphysema, idiopathic pulmonary fibrosis and congenital deficiency of surfactant protein.

In another embodiment, DNA comprises cDNA, which preferably encodes CFTR.

In yet another embodiment, the glycosylated polylysine has a sugar component selected from the group consisting of lactose,  $\alpha$ -glucose,  $\beta$ -galactose, mannose, mannose-6-phosphate, fucose and N-acetylglucosamine. Preferably, the glycosylated polylysine comprises lactosylated polylysine.

In a preferred embodiment, about 10% to about 60% of the amino groups of the polylysine have a lactose molecule substituted thereon. More preferably, about 12% to about 40% of the amino groups of the polylysine have a lactose molecule substituted thereon.

In yet another preferred embodiment, the weight to weight ratio of lactosylated polylysine to DNA in the complex is about one to one to about fifteen to one.

There is further provided in the invention a pharmaceutical composition for treatment of a respiratory disease in a human comprising a complex comprising an isolated nucleic acid and a glycosylated polylysine, wherein the isolated nucleic acid comprises antisense DNA capable of inhibiting the expression of a gene, which gene is required for the development of a respiratory disease in a mammal.

In one embodiment of this aspect of the invention, gene is selected from the group consisting of an interleukin gene and a gene affecting leukotriene synthesis. Preferably, the respiratory disease is asthma and the gene is a gene encoding IL-4 or IL-5.

The pharmaceutical composition may further comprise at least one of chloroquine, glycerol and a fusogenic peptide.

a complex comprising an isolated nucleic acid and a glycosylated polylysine, and adding the complex to the airway epithelial cells.

In one aspect, the composition further comprises at least one of chloroquine, glycerol and a fusogenic peptide.

5 In another aspect, the glycosylated polylysine has a sugar component selected from the group consisting of lactose,  $\alpha$ -glucose,  $\beta$ -galactose, mannose, mannose-6-phosphate, fucose and N-acetylglucosamine. Preferably, the glycosylated polylysine is lactosylated polylysine.

10 In one embodiment, the cells are transfected *in vitro* and in another embodiment, the cells are transfected *in vivo*.

In yet other embodiments, the isolated nucleic acid is DNA or cDNA. The DNA may be selected from the group consisting of DNA encoding CFTR, an asthma gene, DNA encoding  $\alpha$ 1AT, a gene affecting idiopathic pulmonary fibrosis, DNA encoding SP-B and DNA encoding SP-C. Preferably,  
15 the DNA encodes CFTR.

In another embodiment, the DNA is antisense DNA capable of inhibiting the expression of a gene, which gene is required for the development of a respiratory disease in a mammal.

In preferred embodiments, the gene is selected from the group  
20 consisting of an interleukin gene and a gene affecting leukotriene synthesis. More preferably, the respiratory disease is asthma and the gene is a gene encoding IL-4 or a gene encoding IL-5.

The invention also includes a pharmaceutical composition for treatment of a respiratory disease in a human. The composition comprises a  
25 complex comprising an isolated nucleic acid encoding a protein, or a biologically active fragment thereof, and a glycosylated polylysine, wherein the isolated nucleic acid is DNA selected from the group consisting of DNA encoding CFTR, an asthma gene, DNA encoding  $\alpha$ 1AT, a gene affecting idiopathic pulmonary fibrosis, DNA encoding SP-B and DNA encoding SP-C, the complex being suspended in a  
30 pharmaceutically acceptable carrier, the complex being capable of transfecting airway epithelial cells when added thereto.

Physiological Reviews 75:749-757) and Noguee et al. (1994, J. Clin. Invest. 93:1860-1863).

As an alternative to virus or lipid mediated gene therapy, it has been reported that substitution of polylysine with lactose residues facilitates a high level of transfection of HepG2 cells via galactose-specific membrane lectins (Midoux *et al.*, 1993, *Nucleic Acids Res.* **21**:871-878; Erbacher *et al.*, 1995, *Bioconj. Chem.* **6**:401-410). It is also known that partially gluconoylated polylysine is an efficient vehicle for reporter gene expression in a number of different cell types (Midoux *et al.*, 1995, *International Application Publication No. WO 95/30020*; U.S. Patent No. 5,595,897).

Polylysine substituted with specific sugars such as mannose or fucose may be used to transfect human macrophages which have a membrane lectin for mannose and fucose (Erbacher *et al.*, 1996, *Hum. Gene Ther.* **7**:721-729). Further, complex asialo-oligosaccharides coupled to short polylysine polymers have been used to transfect DNA into HepG2 cells (Wadhwa *et al.*, 1995, *Bioconj. Chem.* **6**:283-291).

There remains an acute need for a suitable vehicle for delivery of genes to respiratory cells, which vehicle must be non-immunogenic. Given the paucity of information on the nature of endogenous lectins on human airway epithelial cells (Drickamer *et al.*, 1993, *Ann. Rev. Cell Biol.* **9**:237-264), the use of polylysine derivatized with specific carbohydrates for delivery of genes to airway epithelial cells could not be predicted to successfully facilitate introduction of genes into these cells.

25

#### SUMMARY OF THE INVENTION

The invention relates to a method of transfecting airway epithelial cells comprising adding to the cells a composition comprising a complex comprising an isolated nucleic acid and a glycosylated polylysine.

30

There is also provided in the invention a method of transfecting airway epithelial cells, the method comprising generating a composition comprising

physiological correction of the deficiency. Adenovirus-mediated gene therapy directed to lung cells has been attempted. However, because of the problems associated with adenovirus-induced inflammation, this is not the preferred approach. The use of other viruses and of liposomes has also been contemplated as  
5 a means of delivering  $\alpha$ 1AT to lung cells (Canonica, *supra*).

Idiopathic pulmonary fibrosis (IPF) is a lethal disease with a median time from diagnosis to death of 3 to 5 years. Since, current therapies for IPF have marginal effect on improved lung function or overall survival, a gene therapy approach for treatment of this disease is justified. In IPF, an inflammatory response  
10 to an unidentified insult or injury occurs following an exuberant fibrotic response. The initial inflammatory response is predominantly neutrophilic but evolves to a predominant lymphocytic and monocytic response. As yet, no specific genetic defect has been identified; however, gene therapy targeted to specific sites in the disease pathway, has been contemplated. For example, antisense therapy targeting  
15 specific growth factors or cytokines implicated in IPF has been proposed, in addition to delivery of other genes such as the cyclo-oxygenase-2 gene, the latter of which may block the effects of certain proinflammatory cytokines (Canonica, *supra*).

Congenital deficiency of surfactant protein results in severe  
20 respiratory disease in infants. The fundamental importance of surfactant protein (SP)-B in pulmonary function has been elucidated from studies on infants unable to produce SP-B due a genetic defect which gives rise to a lethal neonatal respiratory disease. Respiratory failure in these infants was refractory to therapies which included mechanical ventilation, surfactant replacement and extracorporeal  
25 membrane oxygenation. A genetically based deficiency in production of a second surfactant protein, SP-C, may also contribute to the development of this disease. Since this disease is governed by genes which have been identified and in view of the absence of any effective current therapy for this disease, a gene therapy approach for treatment of SP-B and/or SP-C deficiency seems appropriate. For a  
30 discussion on congenital deficiency of surfactant protein, see Whitsett et al. (1995,

which ultimately leads to bronchioconstriction. IL-4 induces production by activated B lymphocytes of immunoglobulin (Ig) E which, in turn, induces the production of histamine from mast cells. IL-5 triggers the production by eosinophils of small fatty molecules known as leukotrienes. The combined action  
5 of histamine and leukotrienes causes blood vessels to leak and lung tissues to swell. The smooth muscles of the airways constrict and mucus production is induced which serves to further clog the already constricted airways.

Current asthma therapy is aimed at treating the end result, *i.e.*, the airway constriction. However, targets other than the end point may be more  
10 amenable to therapy, particularly gene therapy. In addition, asthma is believed to have a genetic component, and in fact, the identification of an asthma gene has recently been announced (Vogel, 1997, Science 276:1327). This disease is therefore suitable for treatment using a gene therapy approach.

Alpha<sub>1</sub> antitrypsin ( $\alpha$ 1AT) deficiency, like CF, is an inherited  
15 monogenic disorder having virtually no effective therapy beyond treatment for alleviation of the symptoms of the disease.  $\alpha$ 1AT deficiency is primarily associated with emphysema, a lung disease characterized by unopposed elastolytic destruction of the lung parenchyma. Although  $\alpha$ 1AT is synthesized primarily in liver cells, functional  $\alpha$ 1AT is responsible for over 95% of the antiprotease protection in the  
20 lower respiratory system. The most common genetic abnormality associated with premature emphysema is the Z allele. In this mutant allele, a lysine is substituted for glutamic acid at amino acid position 342 in  $\alpha$ 1AT, thereby altering the three dimensional configuration of the protein and affecting secretion of the protein from the cells in which it is synthesized. Other mutant alleles of the  $\alpha$ 1AT gene also  
25 contribute to the disease, and irrespective of the genetic abnormality, a critical threshold of an  $\alpha$ 1AT serum level of less than 10  $\mu$ M appears necessary for an individual to develop pulmonary emphysema.

Both the liver and the lung have been targeted for gene therapy as a means of treating  $\alpha$ 1AT deficiency. With respect to the liver, although successful  
30 liver-directed  $\alpha$ 1AT gene therapy has been achieved using various strategies, serum  $\alpha$ 1AT levels in all of these systems were below what would be necessary for



most important and life threatening pathology occurs in the lung. Gene therapy has been proposed as a means of developing effective therapy to combat the pathology of CF. However, there are a plethora of problems associated with this approach, not the least of which is the lack of a suitable vehicle for delivery of the CFTR gene to humans.

Initial reports of gene therapy as a means of treating CF have focussed on airway epithelial cells as targets for the CFTR gene. Viral vectors have been used as vehicles for delivery of the CFTR gene to these cells in humans. However, the vectors themselves have proved to be sufficiently immunogenic so as to diminish any positive effect of the successful delivery of the CFTR gene to the affected cells of the individual (Wilson, 1995, *J. Clin. Invest.* **96**:2547-2554; Crystal *et al.*, 1995, *Science* **270**:404-410).

Other vehicles which have been used as gene delivery vehicles include cationic lipids for transfer of genes to airway epithelial cells (Fasbender *et al.*, 1995, *Am. J. Physiol.* **269**:L45-L51). In addition, polylysines (poly-L-lysine) complexed with various glycoproteins, including transferrin targeted to the transferrin receptor, have been examined (Curiel *et al.*, 1991, *Proc. Natl. Acad. Sci. USA* **88**:8850-8854). Also reported, is the use of asialoglycoproteins targeted to hepatic cells through the asialoglycoprotein receptor (Wilson *et al.*, 1992, *J. Biol. Chem.* **267**:963-967), and the use of Tn antigen for gene transfer (Thurnher *et al.*, 1994, *Glycobiology* **4**:429-435). The above-referenced studies have largely been performed in cells other than airway epithelial cells. Moreover, complexes having glycoprotein as a component thereof are potentially immunogenic and therefore may not be of immediate value in human gene therapy.

Asthma is a disease of the industrialized 20th century, being described for the first time in the mid-1800's. Exposure to otherwise harmless pollens and other allergens may set off a life threatening asthma attack in susceptible individuals, wherein constriction of the bronchioles renders a patient virtually unable to breathe. Asthma attacks are triggered by exposure to allergens which cause activated T lymphocytes of the  $T_H2$  subset to secrete cytokines, primarily interleukin 4 (IL-4) and interleukin 5 (IL-5) setting off a cascade of events

## NON-VIRAL VEHICLES FOR USE IN GENE TRANSFER

## FIELD OF THE INVENTION

5           The field of the invention is delivery of genes to cells *in vitro* and *in vivo*, in particular, non-viral delivery of genes to cells.

## GOVERNMENT SUPPORT

          Portions of this invention were supported by a grant from the U.S.  
10   Government (NIH Grant No. RO1 16859) and the U.S. Government may therefore have certain rights in the invention.

## BACKGROUND OF THE INVENTION

          Diseases of the respiratory tract are among the most common  
15   diseases in humans and range in severity from being merely mild and annoying to life threatening. Examples of severe to life threatening respiratory diseases in humans include cystic fibrosis, asthma, emphysema, idiopathic pulmonary fibrosis and congenital deficiency of surfactant protein. Each of these diseases is suitable  
20   for gene therapy as a means of treatment provided that a gene delivery vehicle is available which delivers the appropriate gene in an effective and non-toxic manner. Gene therapy approaches which have been used or are contemplated for treatment of these respiratory diseases are reviewed in Canonico (1997, *Gene Therapy for Chronic Inflammatory Diseases of the Lungs in Gene Therapy for Diseases of the Lung*, K.L. Brigham, ed, Marcel Dekker, New York, pp.285-307).

25           Cystic fibrosis (CF) is the most common lethal genetic disease in Caucasians and, although the average life expectancy has increased to approximately 30 years in the past decade, there remains no effective cure for CF (Scanlin *et al.*, 1988, A.P. Fishman, ed. (McGraw-Hill, New York) pp. 1273-1294; Welsh *et al.*, 1995, Scriber *et al.*, eds. (McGraw-Hill, New York) pp. 3799-3876).

30           Patients having CF encode a mutated cystic fibrosis transmembrane conductance regulator (CFTR) gene. Although CF is a multisystem disease, the

**FOR THE PURPOSES OF INFORMATION ONLY**

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	ES	Spain	LS	Lesotho	SI	Slovenia
AM	Armenia	FI	Finland	LT	Lithuania	SK	Slovakia
AT	Austria	FR	France	LU	Luxembourg	SN	Senegal
AU	Australia	GA	Gabon	LV	Latvia	SZ	Swaziland
AZ	Azerbaijan	GB	United Kingdom	MC	Monaco	TD	Chad
BA	Bosnia and Herzegovina	GE	Georgia	MD	Republic of Moldova	TG	Togo
BB	Barbados	GH	Ghana	MG	Madagascar	TJ	Tajikistan
BE	Belgium	GN	Guinea	MK	The former Yugoslav	TM	Turkmenistan
BF	Burkina Faso	GR	Greece		Republic of Macedonia	TR	Turkey
BG	Bulgaria	HU	Hungary	ML	Mali	TT	Trinidad and Tobago
BJ	Benin	IE	Ireland	MN	Mongolia	UA	Ukraine
BR	Brazil	IL	Israel	MR	Mauritania	UG	Uganda
BY	Belarus	IS	Iceland	MW	Malawi	US	United States of America
CA	Canada	IT	Italy	MX	Mexico	UZ	Uzbekistan
CF	Central African Republic	JP	Japan	NE	Niger	VN	Viet Nam
CG	Congo	KE	Kenya	NL	Netherlands	YU	Yugoslavia
CH	Switzerland	KG	Kyrgyzstan	NO	Norway	ZW	Zimbabwe
CI	Côte d'Ivoire	KP	Democratic People's	NZ	New Zealand		
CM	Cameroon		Republic of Korea	PL	Poland		
CN	China	KR	Republic of Korea	PT	Portugal		
CU	Cuba	KZ	Kazakstan	RO	Romania		
CZ	Czech Republic	LC	Saint Lucia	RU	Russian Federation		
DE	Germany	LI	Liechtenstein	SD	Sudan		
DK	Denmark	LK	Sri Lanka	SE	Sweden		
EE	Estonia	LR	Liberia	SG	Singapore		

**PCT**WORLD INTELLECTUAL PROPERTY ORGANIZATION  
International Bureau

## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

<b>(51) International Patent Classification <sup>6</sup> :</b> <b>C12P 21/06, C12N 5/00, 15/00, C07H 21/02, 21/04, A01N 43/04</b>		<b>A1</b>	<b>(11) International Publication Number:</b> <b>WO 98/06869</b>
			<b>(43) International Publication Date:</b> 19 February 1998 (19.02.98)
<b>(21) International Application Number:</b> PCT/US97/14280		<b>(81) Designated States:</b> AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, HU, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, UZ, VN, YU, ZW, ARIPO patent (GH, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).	
<b>(22) International Filing Date:</b> 13 August 1997 (13.08.97)			
<b>(30) Priority Data:</b> 60/023,941 14 August 1996 (14.08.96) US 08/907,673 8 August 1997 (08.08.97) US			
<b>(71) Applicant:</b> THE CHILDREN'S HOSPITAL OF PHILADELPHIA [US/US]; 34th Street & Civic Center Boulevard, Philadelphia, PA 19104-4318 (US).			
<b>(72) Inventors:</b> GLICK, Mary, Catherine; 2028 Delancey Place, Philadelphia, PA 19104 (US). SCANLIN, Thomas, F.; 4250 Regent Square, Philadelphia, PA 19104 (US). KOLLEN, Wouter, J., W.; 415 South Van Pelt Street B-2, Philadelphia, PA 19146 (US).			
<b>(74) Agents:</b> LEARY, Kathryn, Doyle et al.; Panitch Schwarze Jacobs & Nadel, P.C., 36th floor, 1601 Market Street, Philadelphia, PA 19103-2398 (US).		<b>Published</b> <i>With international search report.</i> <i>Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>	
<b>(54) Title:</b> NON-VIRAL VEHICLES FOR USE IN GENE TRANSFER			
<b>(57) Abstract</b> <p>The invention relates to compositions and methods for transfection of cells, particularly airway epithelial cells, with DNA complexed to polylysine substituted with glycosyl residues. The invention also relates to methods of treating humans having respiratory disease comprising administering to a human the composition of the invention.</p>			

INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US97/14280

A. CLASSIFICATION OF SUBJECT MATTER:

IPC (6):

C12P 21/06; C12N 5/00, 15/00; C07H 21/02, 21/04; A01N 43/04

A. CLASSIFICATION OF SUBJECT MATTER:

US CL :

435/6, 69.1, 172.1, 172.3; 514/2, 44; 536/23.1, 23.2, 23.4, 320.1, 325

## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US97/14280

## C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	ERBACHER, P. et al. Glycosylated Polylysine/DNA Complexes: Gene Transfer Efficiency in Relation with the Size and the Sugar Substitution Level of Glycosylated Polylysines and with the Plasmid Size. Bioconjugate Chemistry. 1995, Vol. 6, No. 4, pages 401-410, see entire document.	1-77

## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US97/14280

## A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) : Please See Extra Sheet.

US CL : Please See Extra Sheet.

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/6, 69.1, 172.1, 172.3; 514/2, 44; 536/23.1, 23.2, 23.4, 320.1, 325

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched  
NONEElectronic data base consulted during the international search (name of data base and, where practicable, search terms used)  
APS, MEDLINE, BIOSIS, SCISEARCH, EMBASE, CAPLUS

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	ERBACHER, P. et al. Putative Roles of Chloroquine in Gene Transfer into Human Hepatoma Cell Line by DNA/Lactosylated Polylysine Complexes. Experimental Cell Research. 1996, Vol. 225, pages 186-194, see entire document.	1-77
Y	ERBACHER, P. et al. Gene Transfer by DNA/Glycosylated Polylysine Complexes into Human Blood Monocyte-Derived Macrophages. Human Gene Therapy. 10 April 1996, Vol. 7, pages 721-729, see entire document.	1-77
Y	MIDOUX, P. et al. Specific Gene Transfer Mediated by Lactosylated Poly-L-Lysine into Hepatoma Cells. Nucleic Acids Research. 1993, Vol. 21, No. 4, pages 871-878, see entire document.	1-77

☒ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:	*T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
*A* document defining the general state of the art which is not considered to be of particular relevance	*X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
*B* earlier document published on or after the international filing date	*Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
*L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	*A* document member of the same patent family
*O* document referring to an oral disclosure, use, exhibition or other means	
*P* document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search

29 SEPTEMBER 1997

Date of mailing of the international search report

14 JAN 1998

Name and mailing address of the ISA/US  
Commissioner of Patents and Trademarks  
Box PCT  
Washington, D.C. 20231

Facsimile No. (703) 305-3230

Authorized officer

SEAN MCGARRY

Telephone No. (703) 308-0196

Fig. 18A

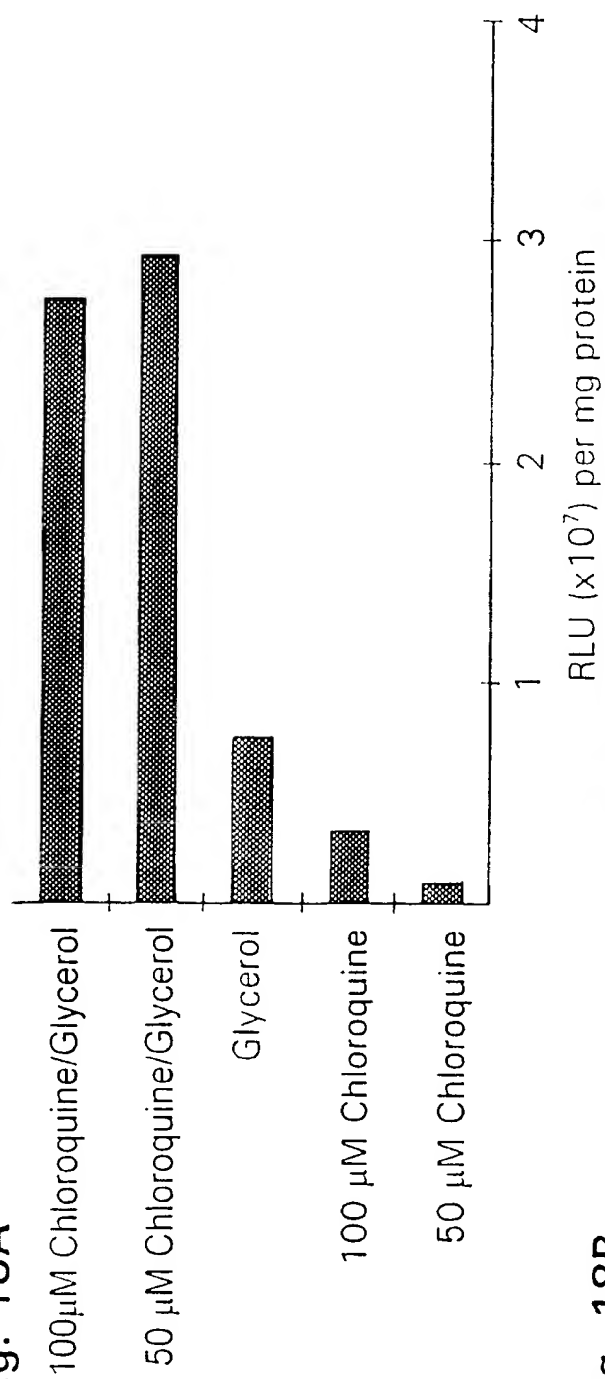
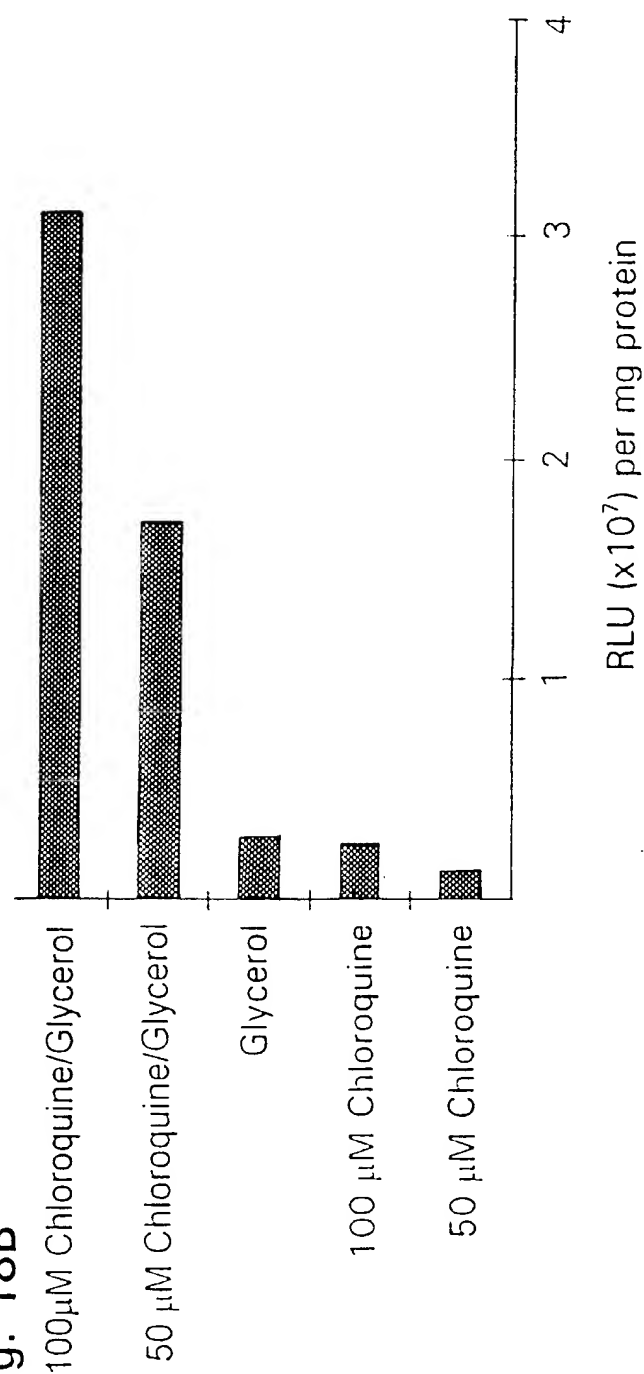
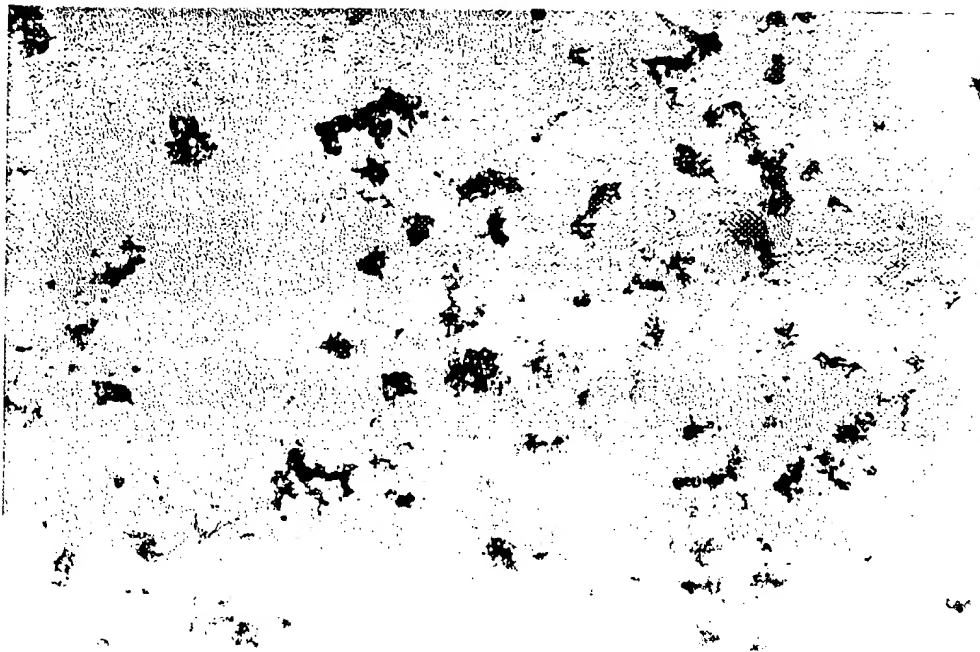


Fig. 18B

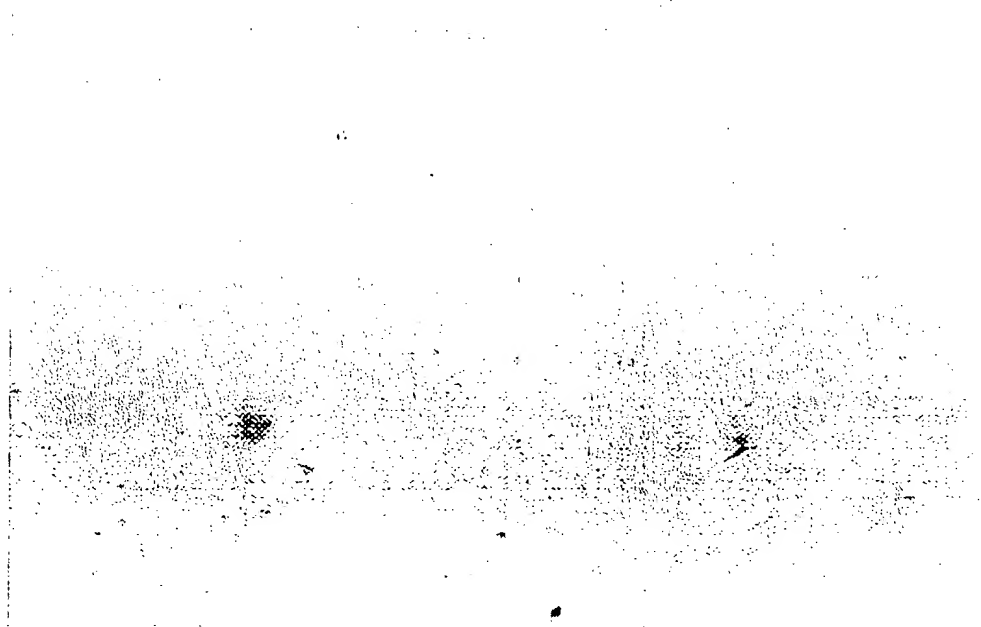




**FIGURE 17A**



**FIGURE 17B**



**FIGURE 16C**

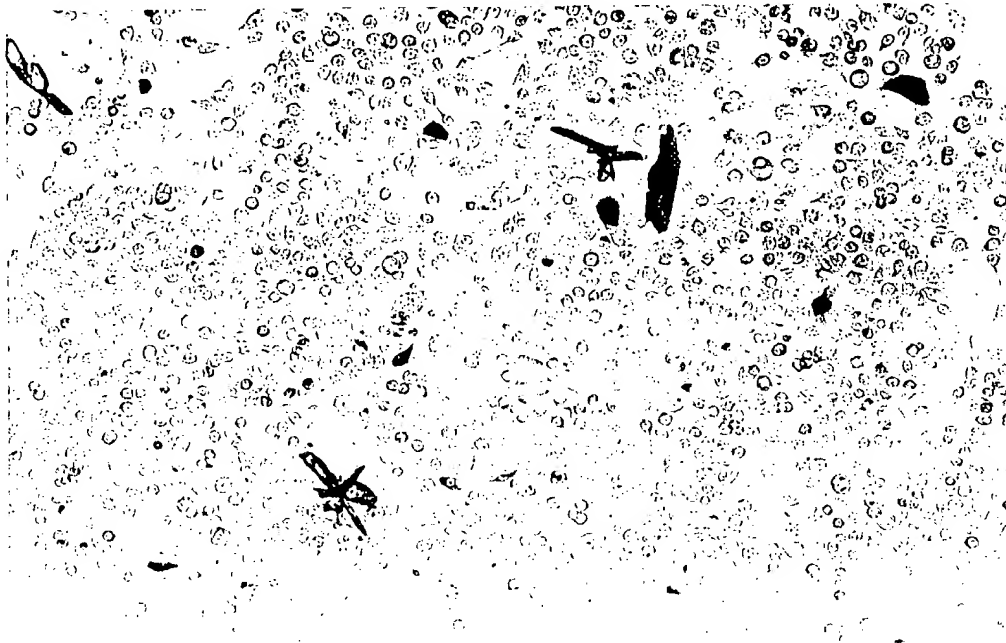


FIGURE 16A

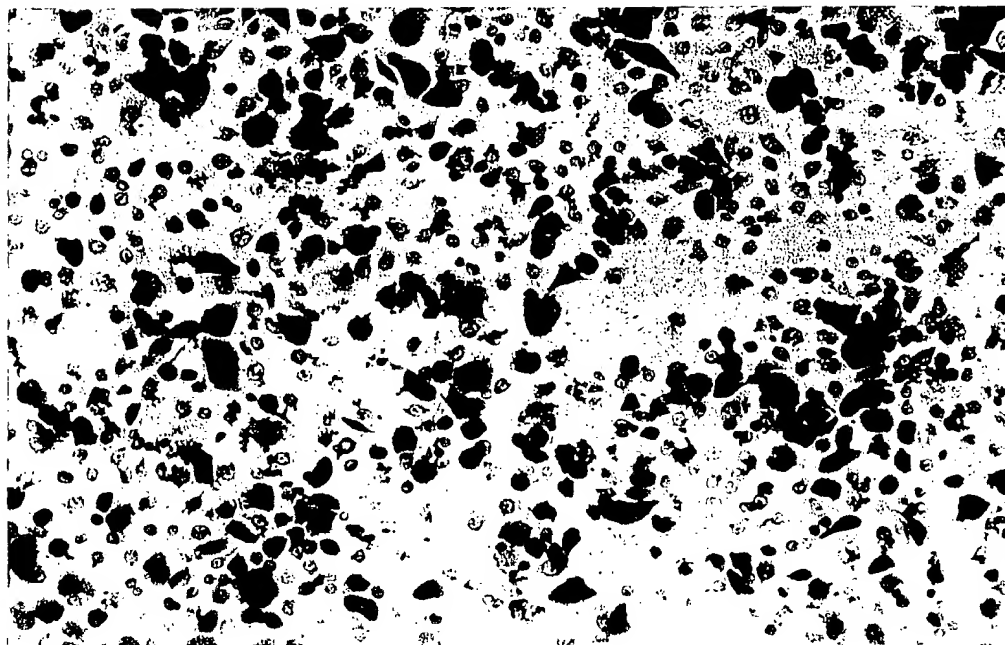


FIGURE 16B

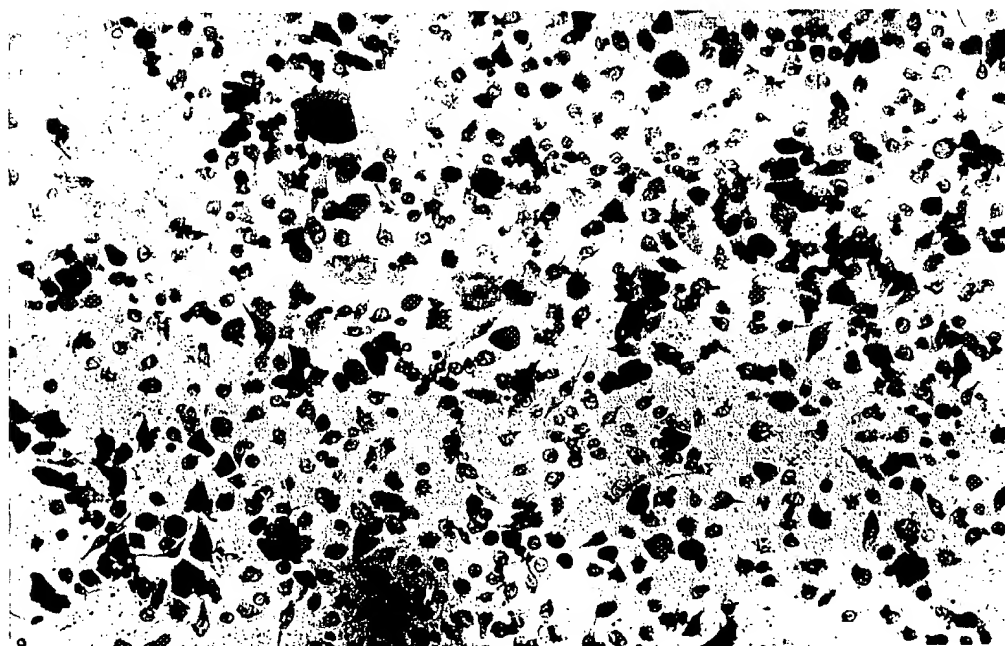


Fig. 15A

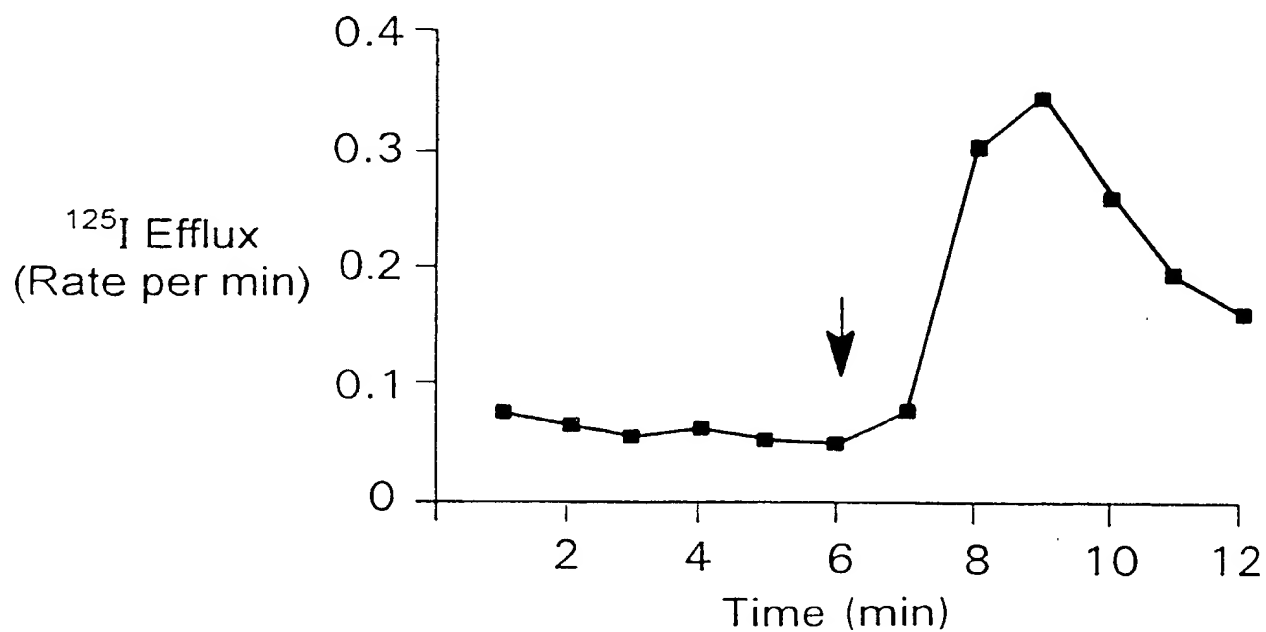


Fig. 15B

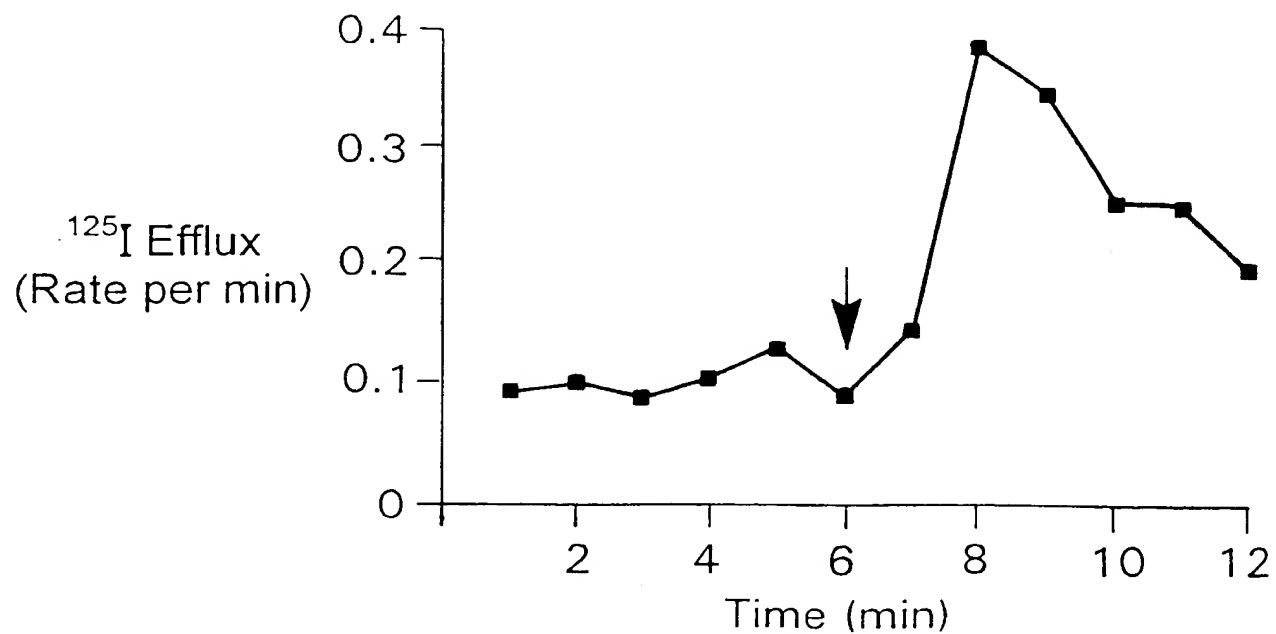


Fig. 14

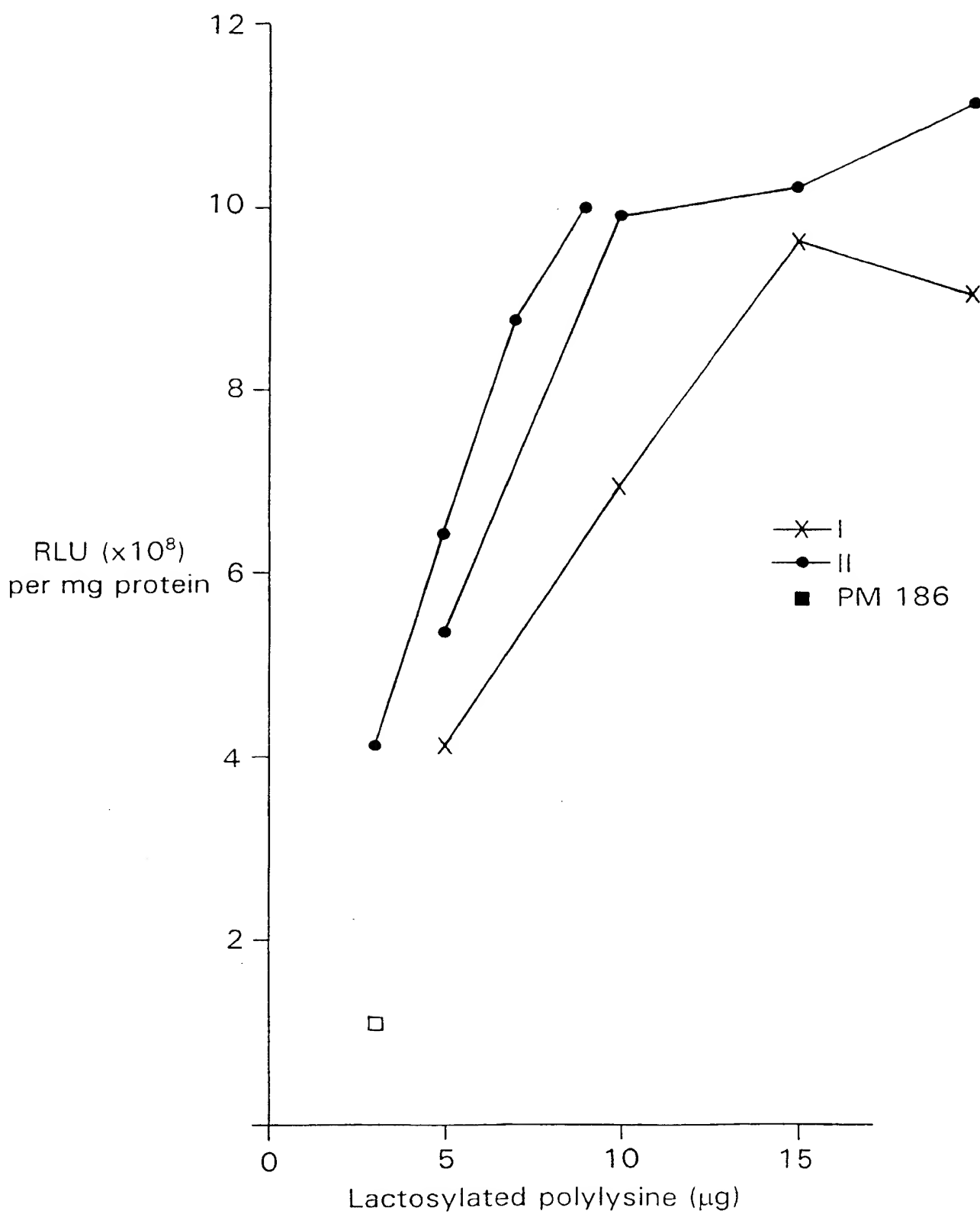


Fig. 13

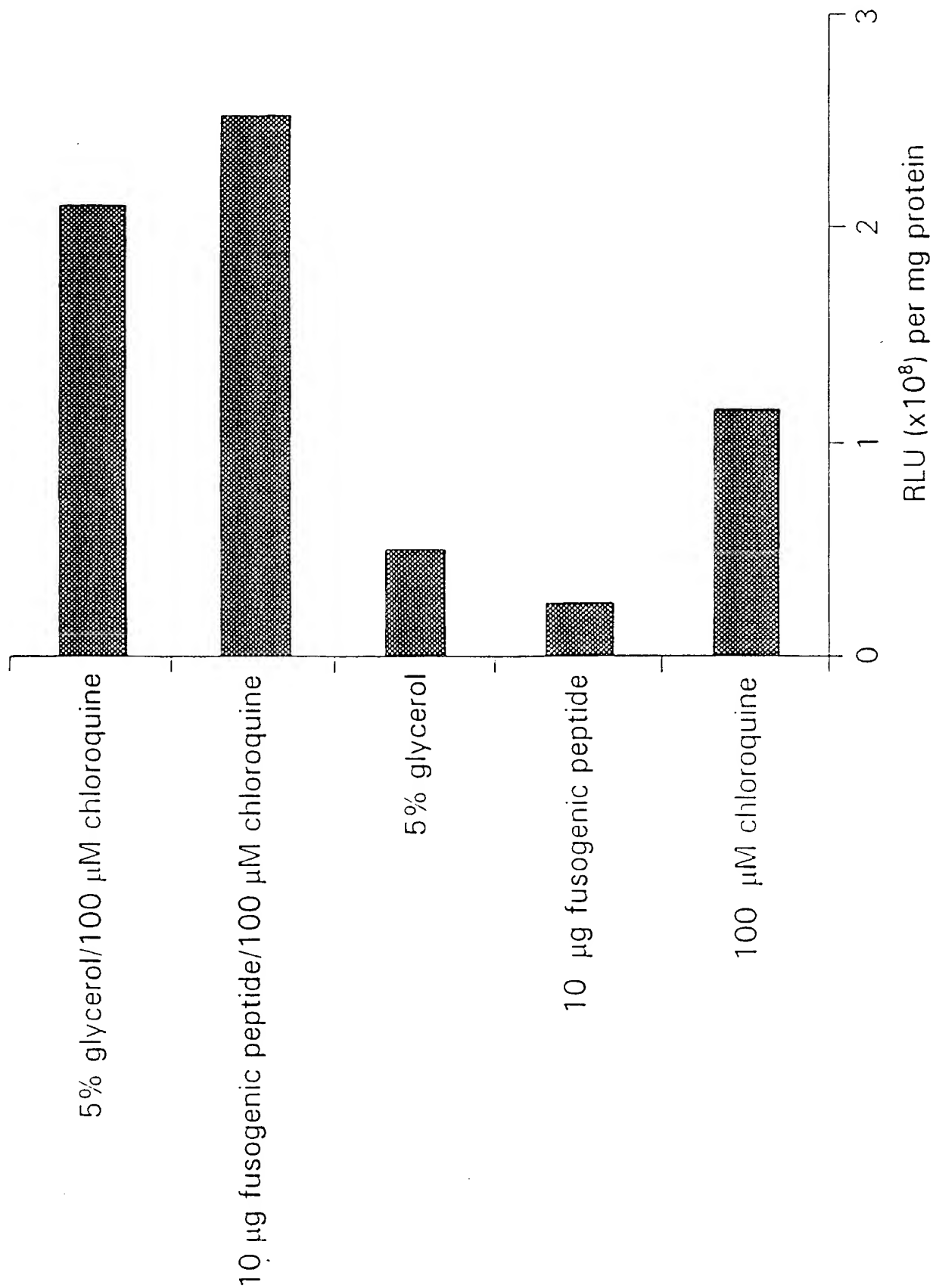


Fig. 12

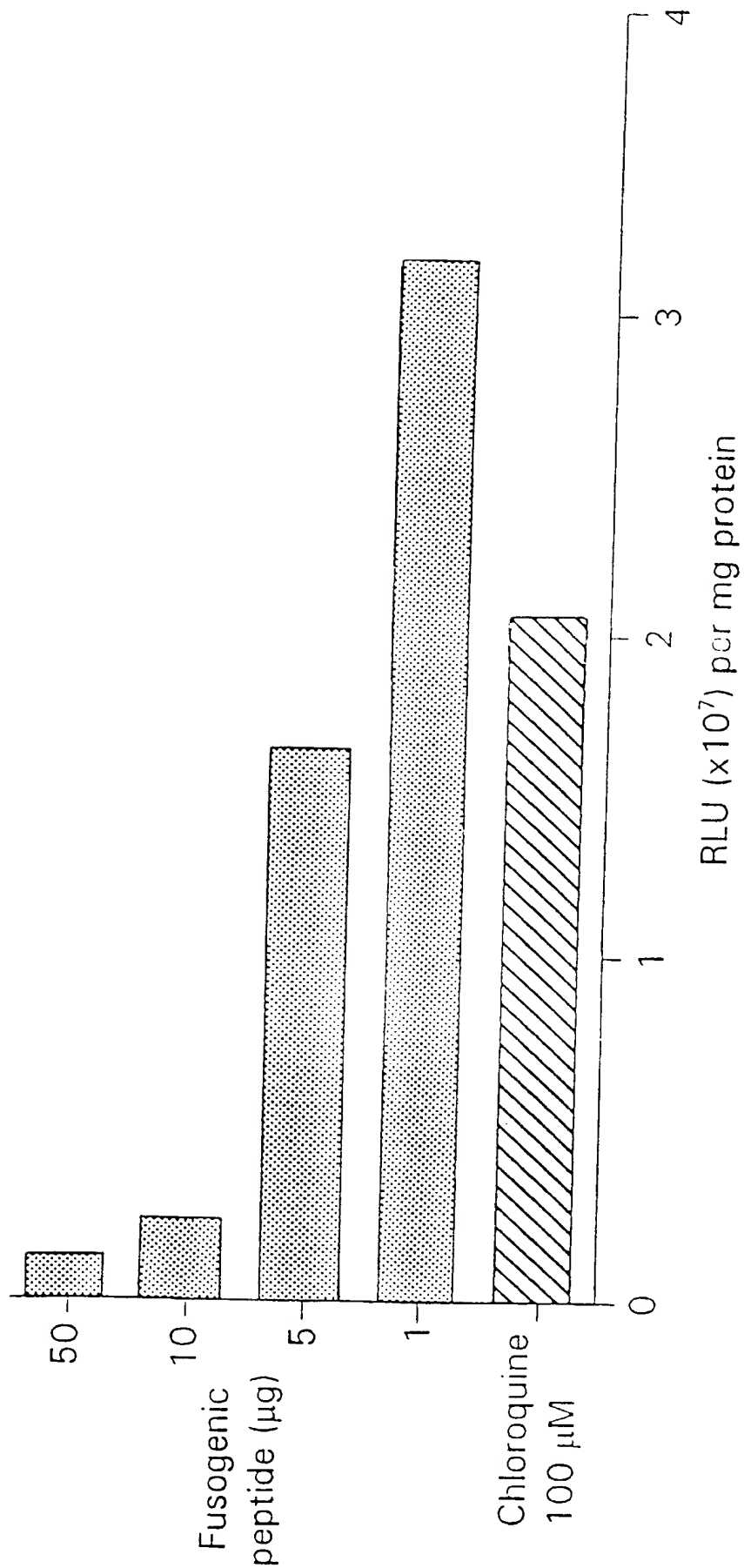


Fig. 11

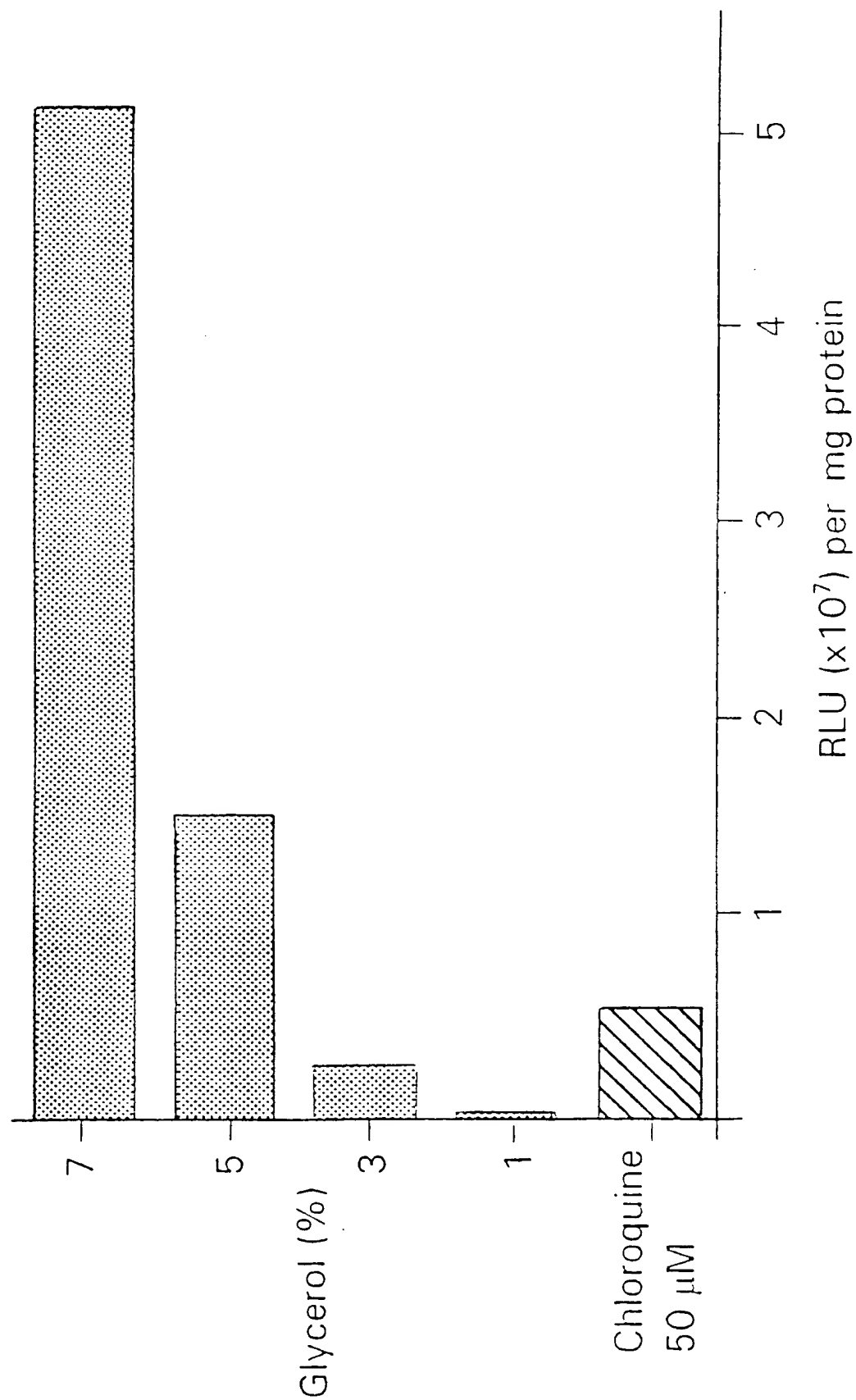
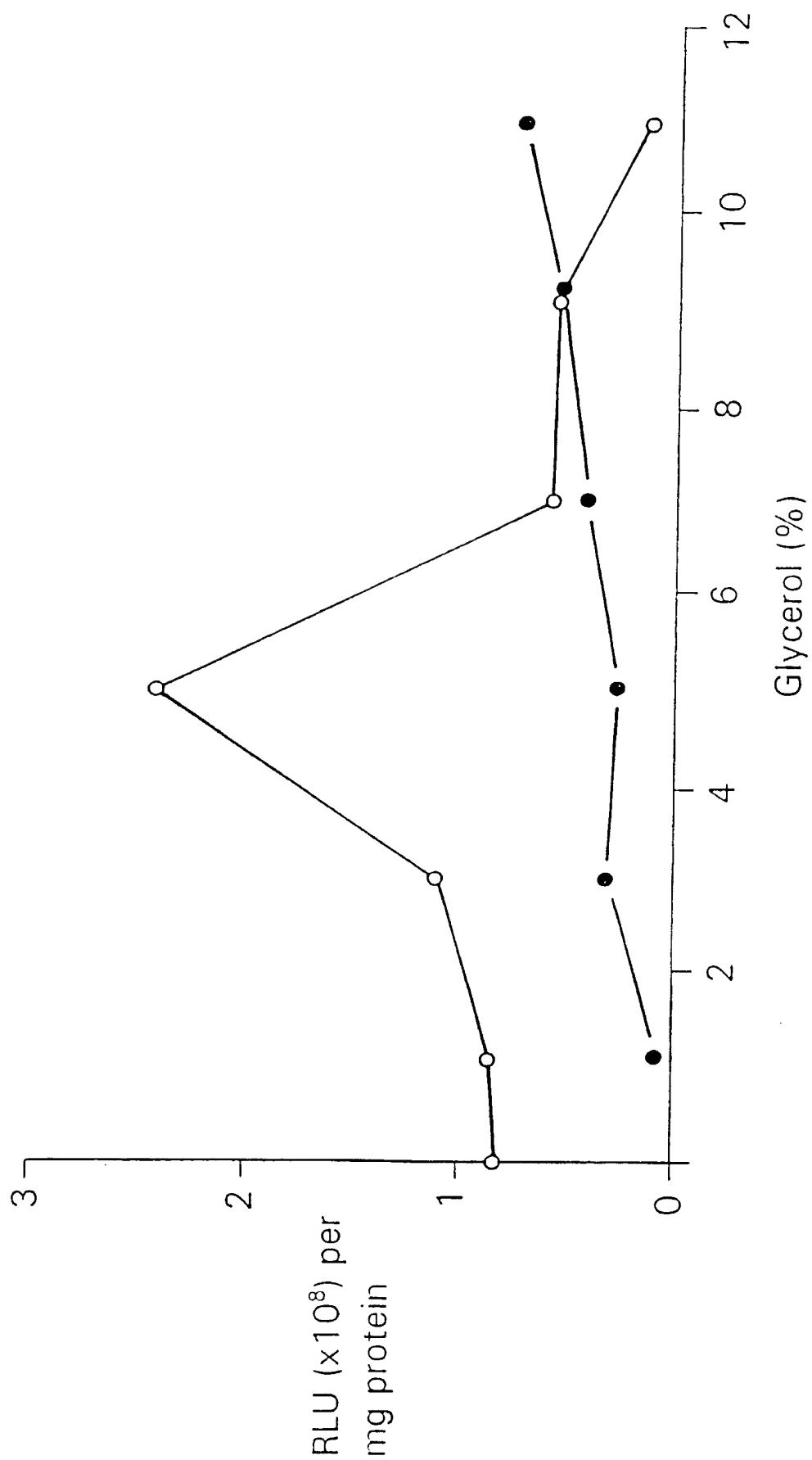
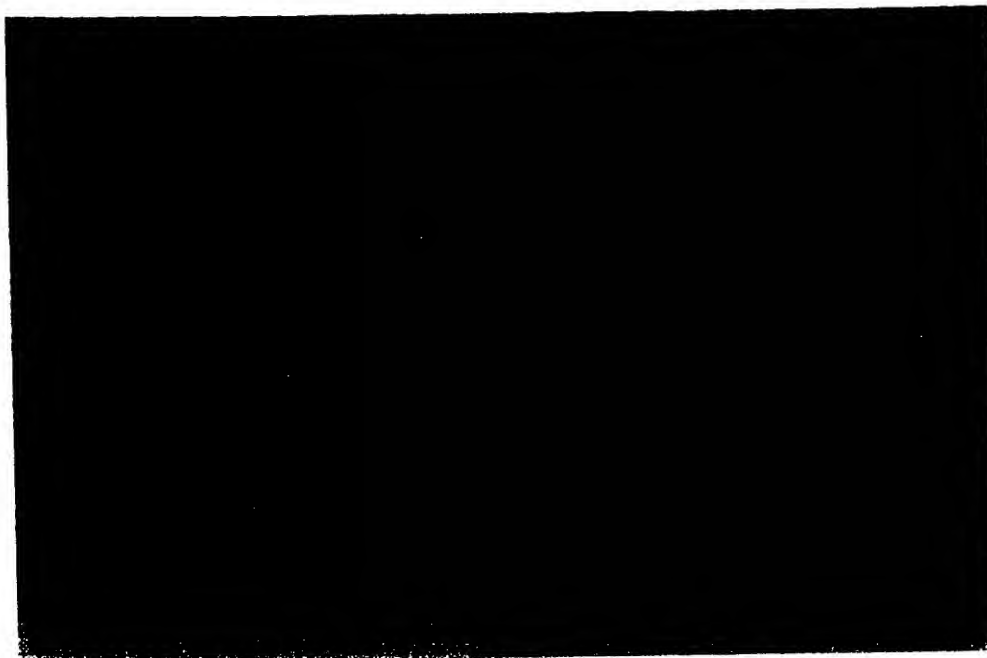




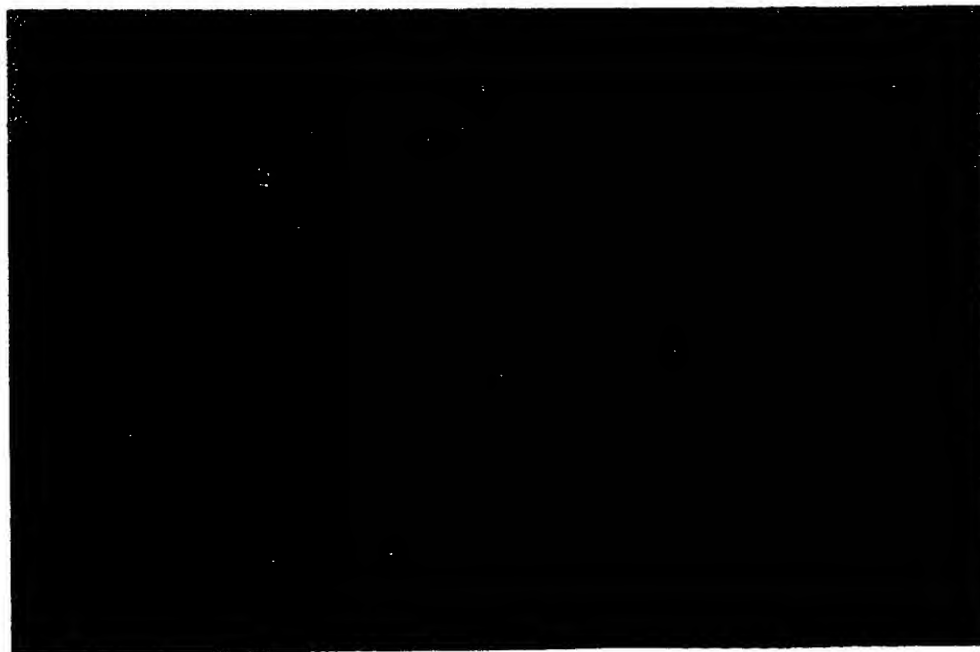
Fig. 10

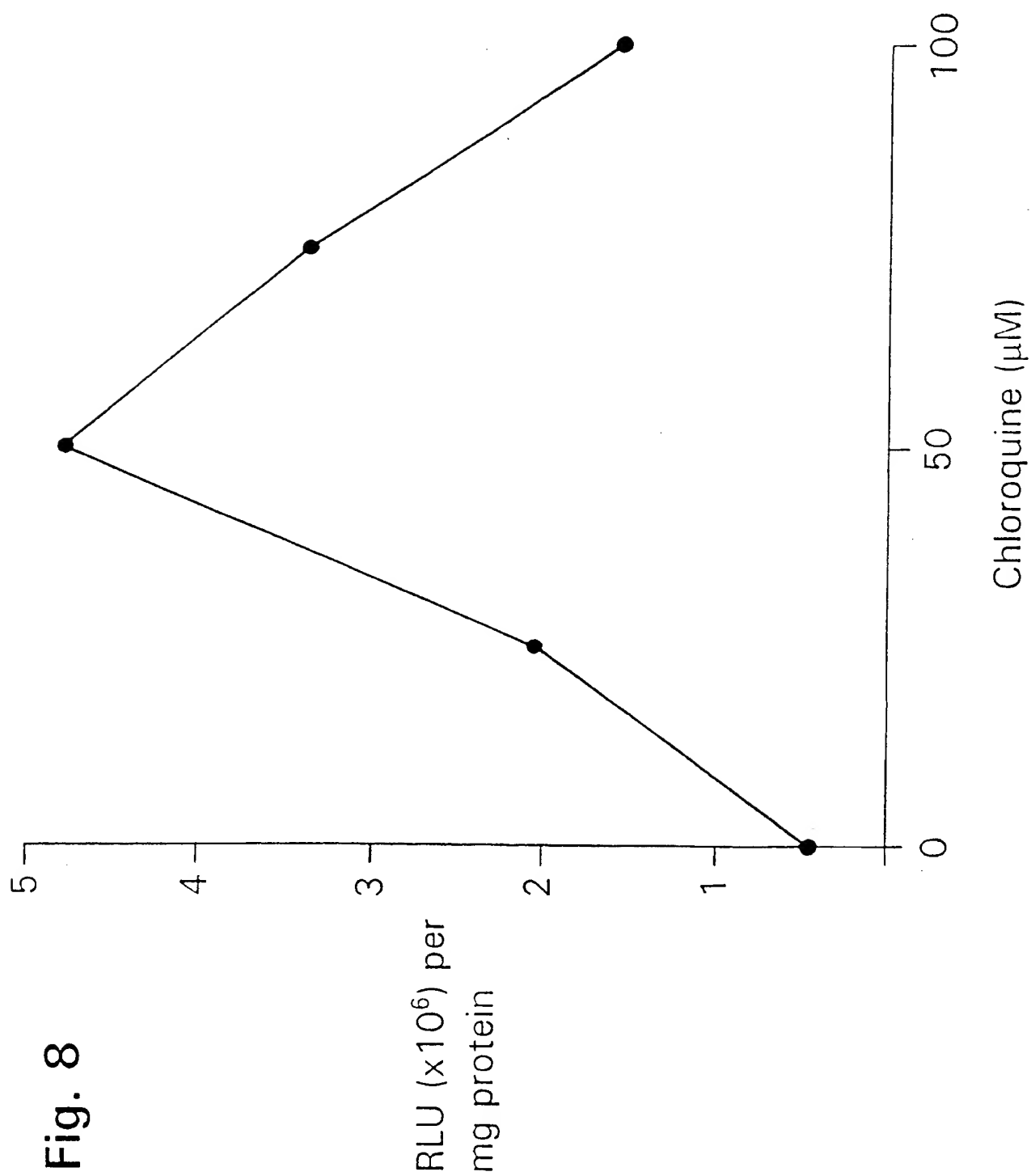


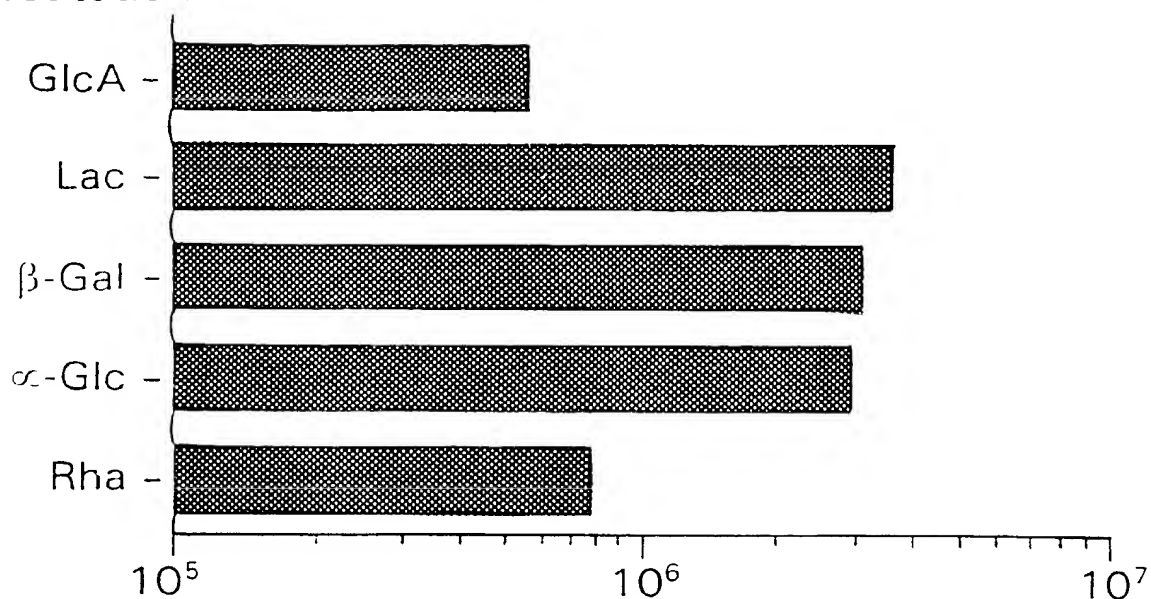
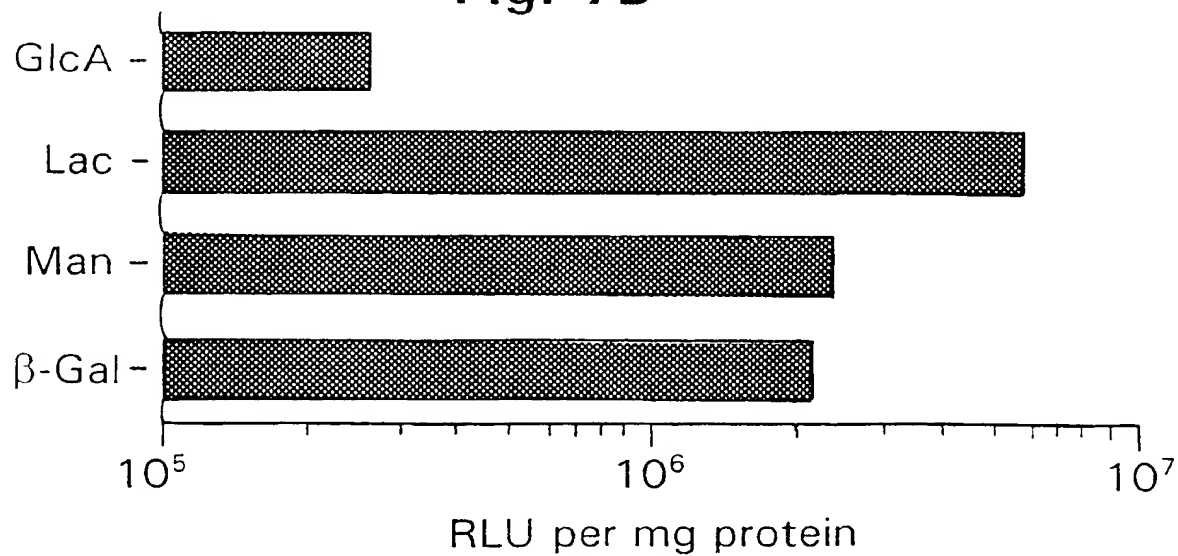
**FIGURE 9A**



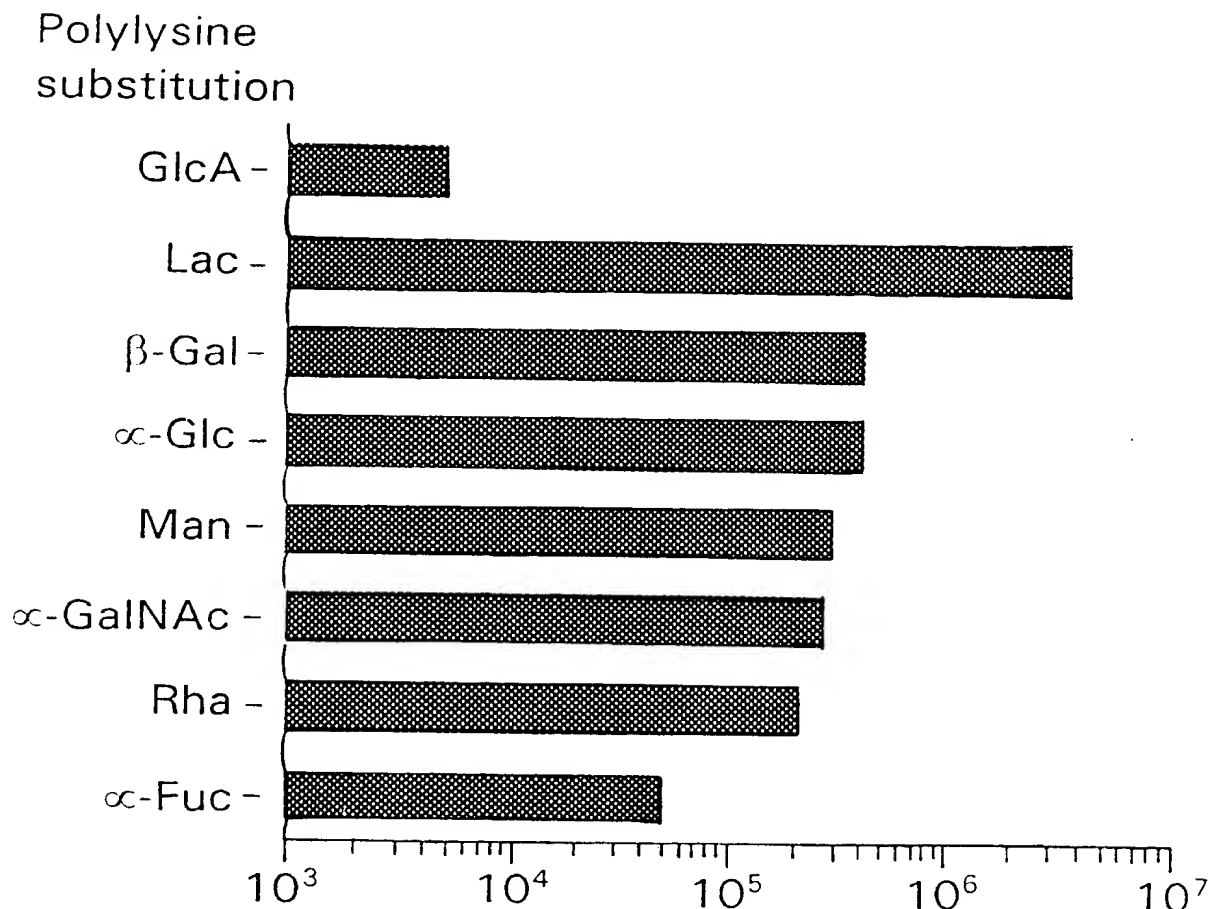
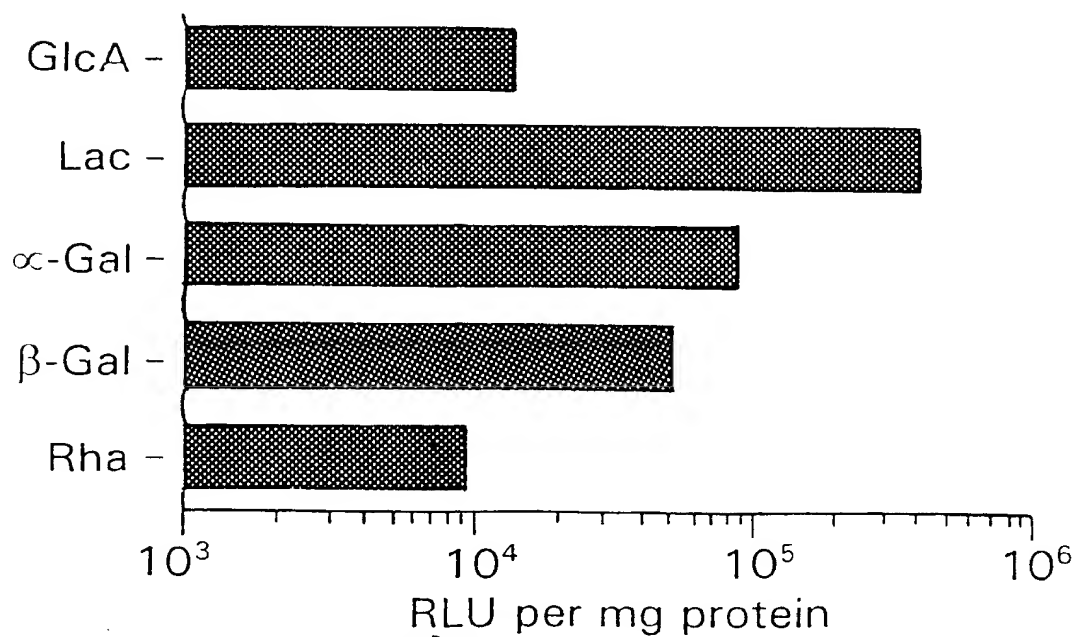
**FIGURE 9B**

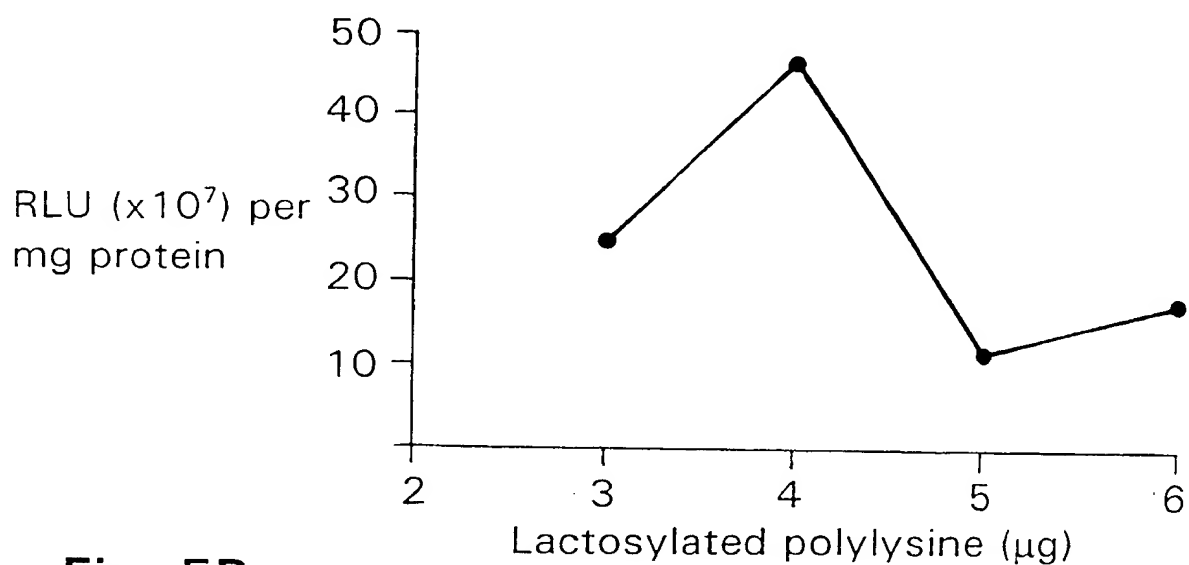
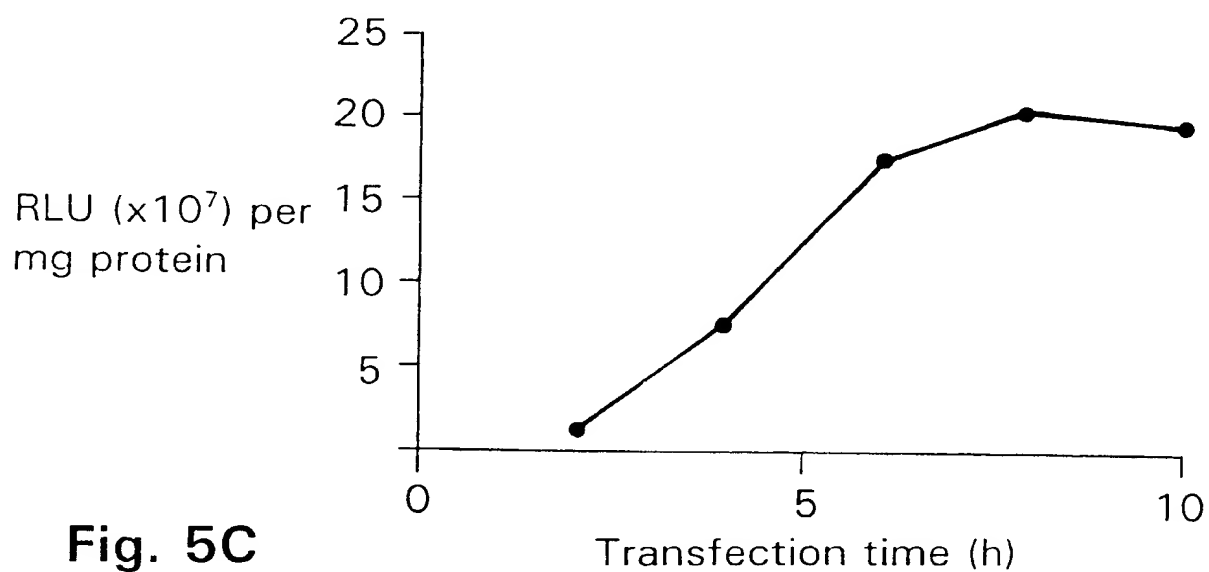
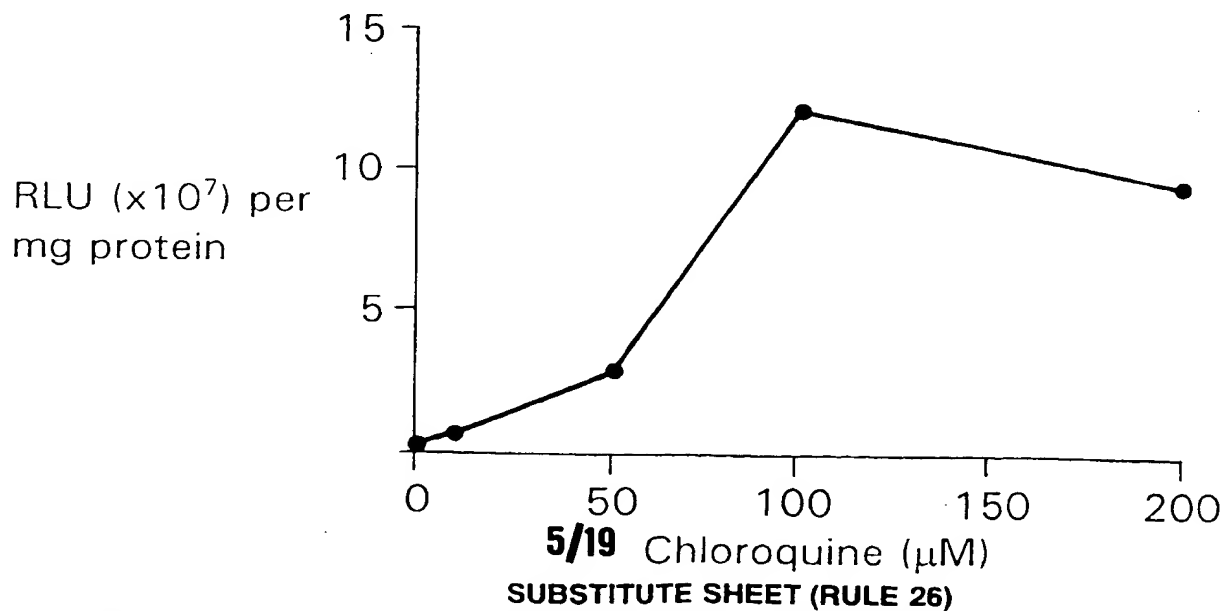




**Fig. 7A**Polylysine  
substitution**Fig. 7B**

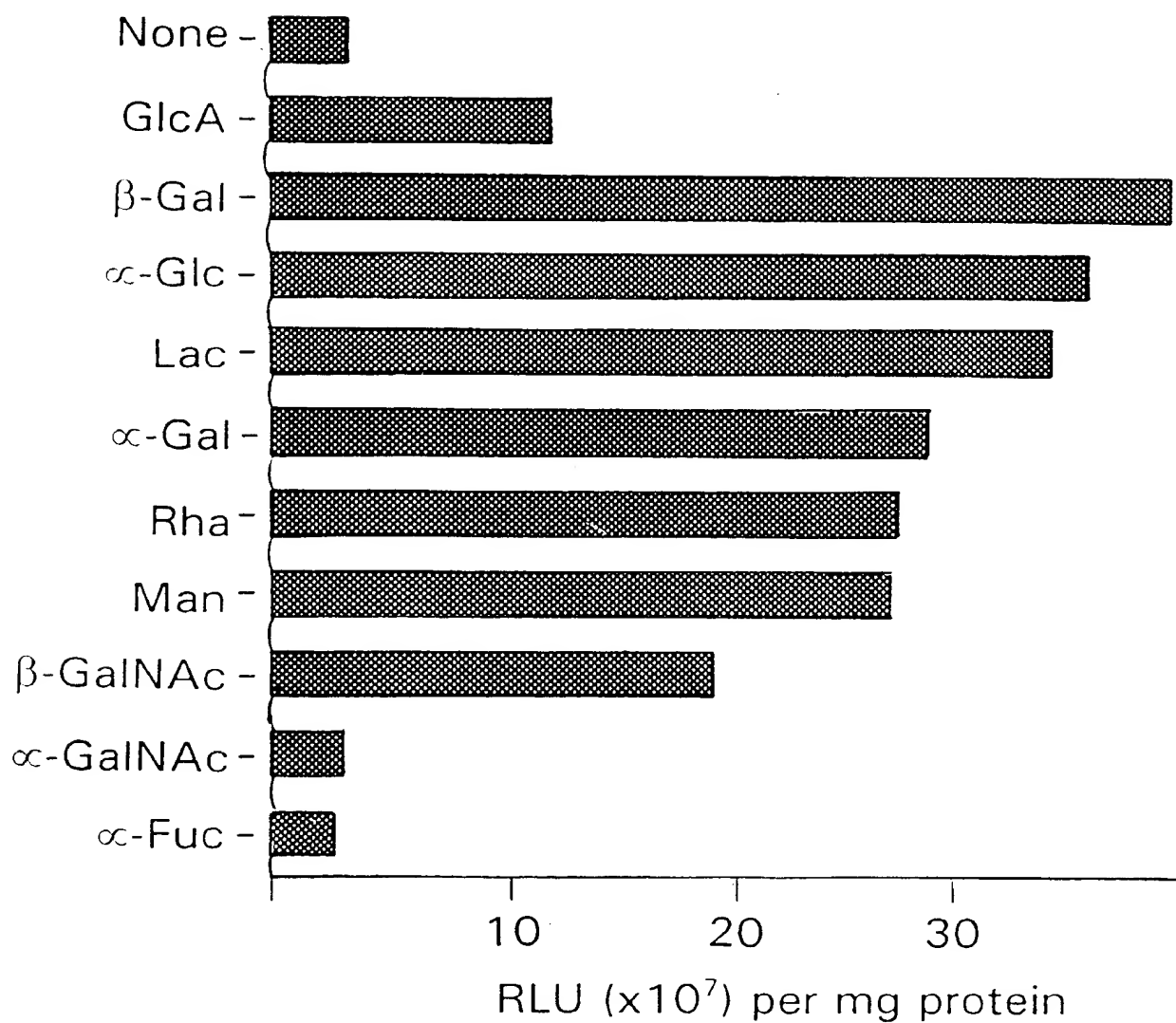
RLU per mg protein

**Fig. 6A****Fig. 6B**

**Fig. 5A****Fig. 5B****Fig. 5C**

SUBSTITUTE SHEET (RULE 26)

Polylysine  
substitution



**Fig. 4**

Fig. 3A

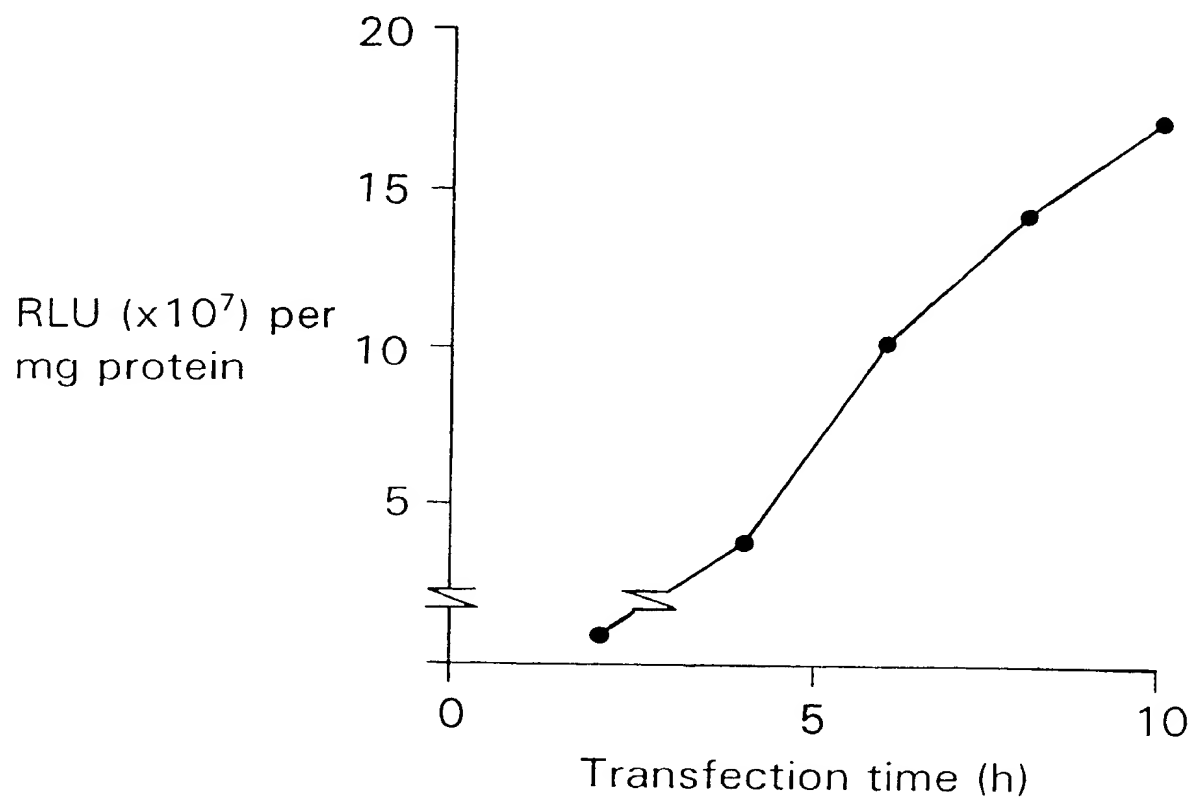


Fig. 3B

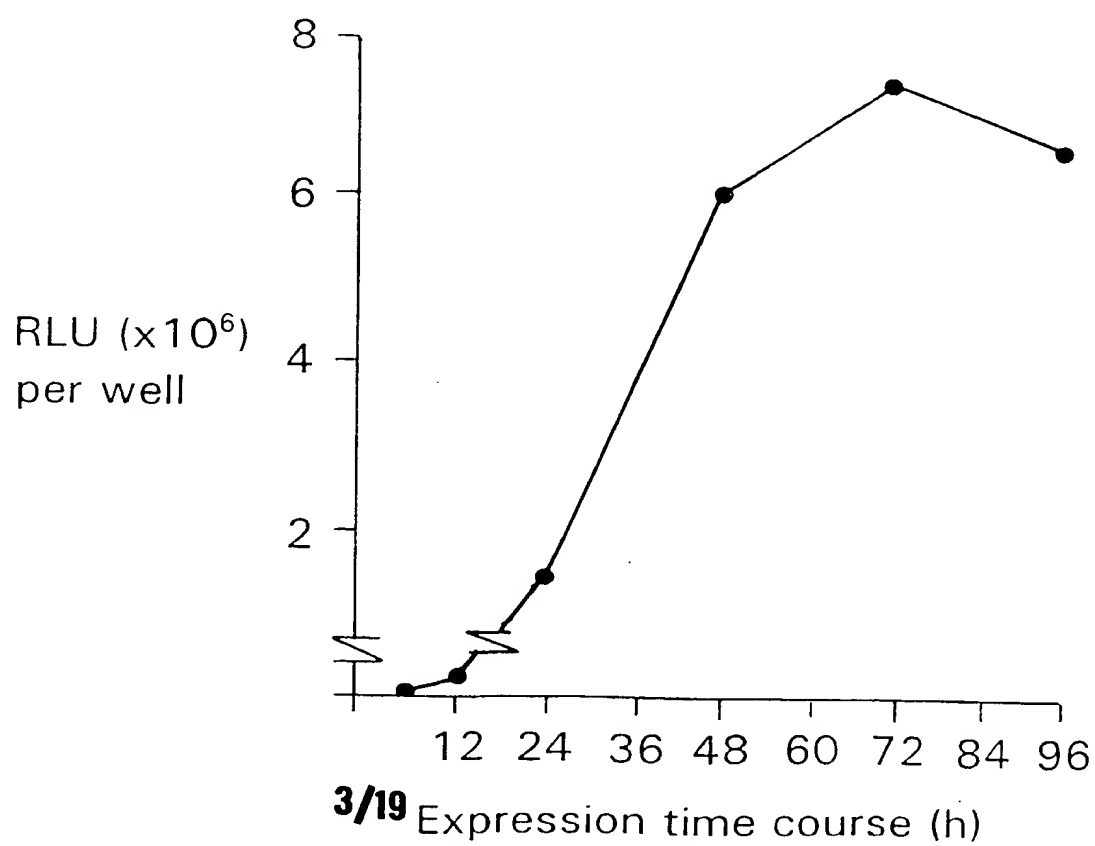




Fig. 2A

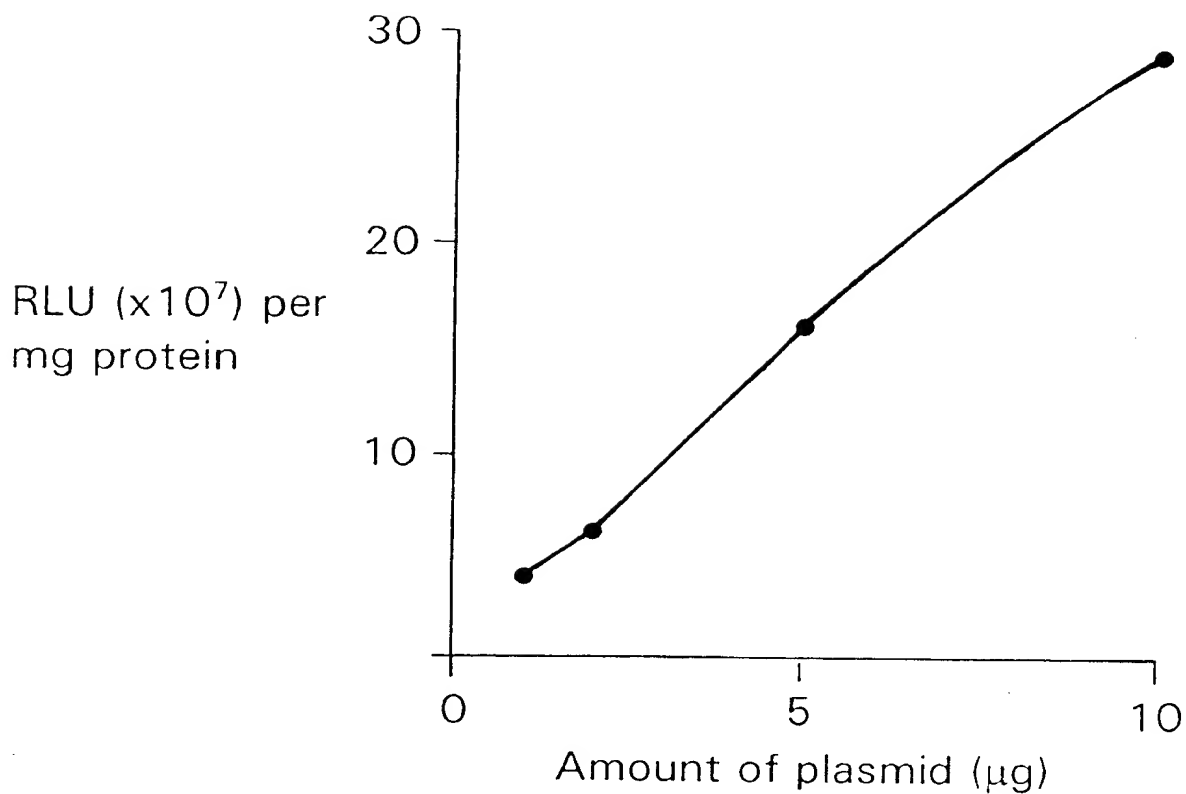
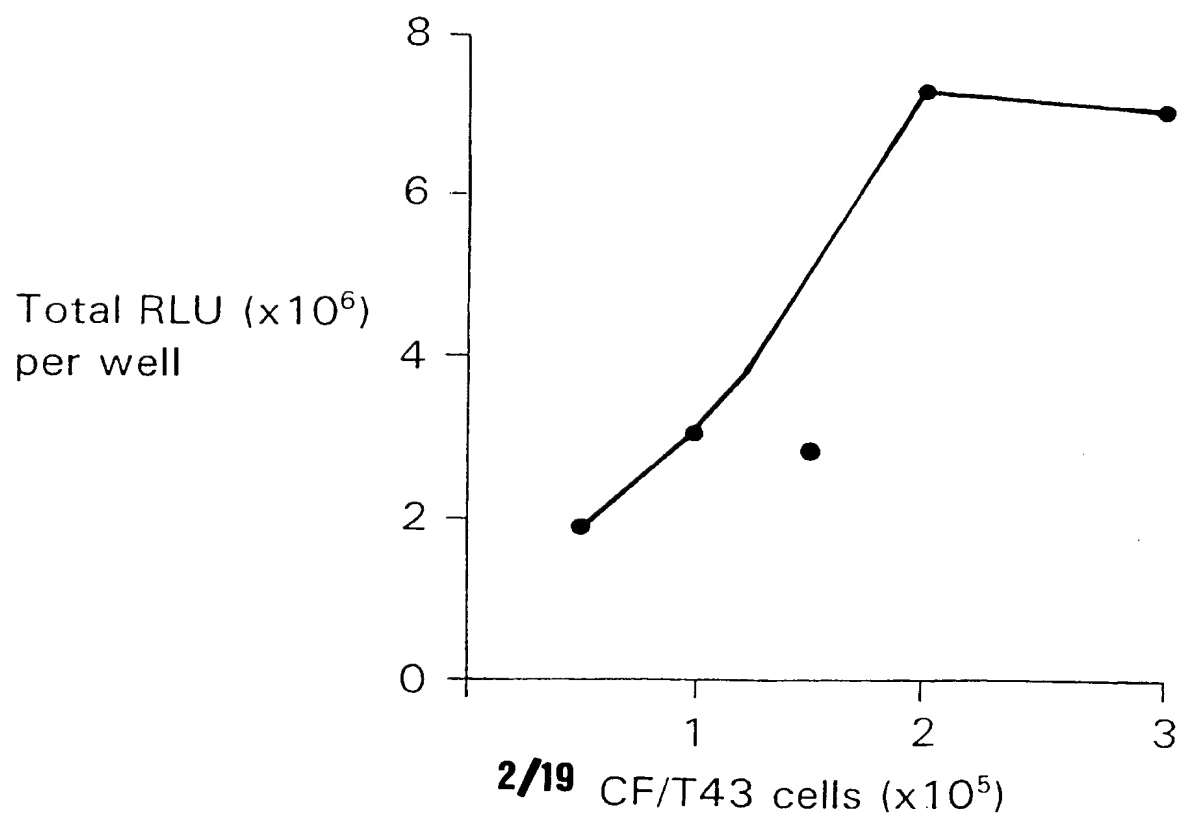


Fig. 2B



SUBSTITUTE SHEET (RULE 26)

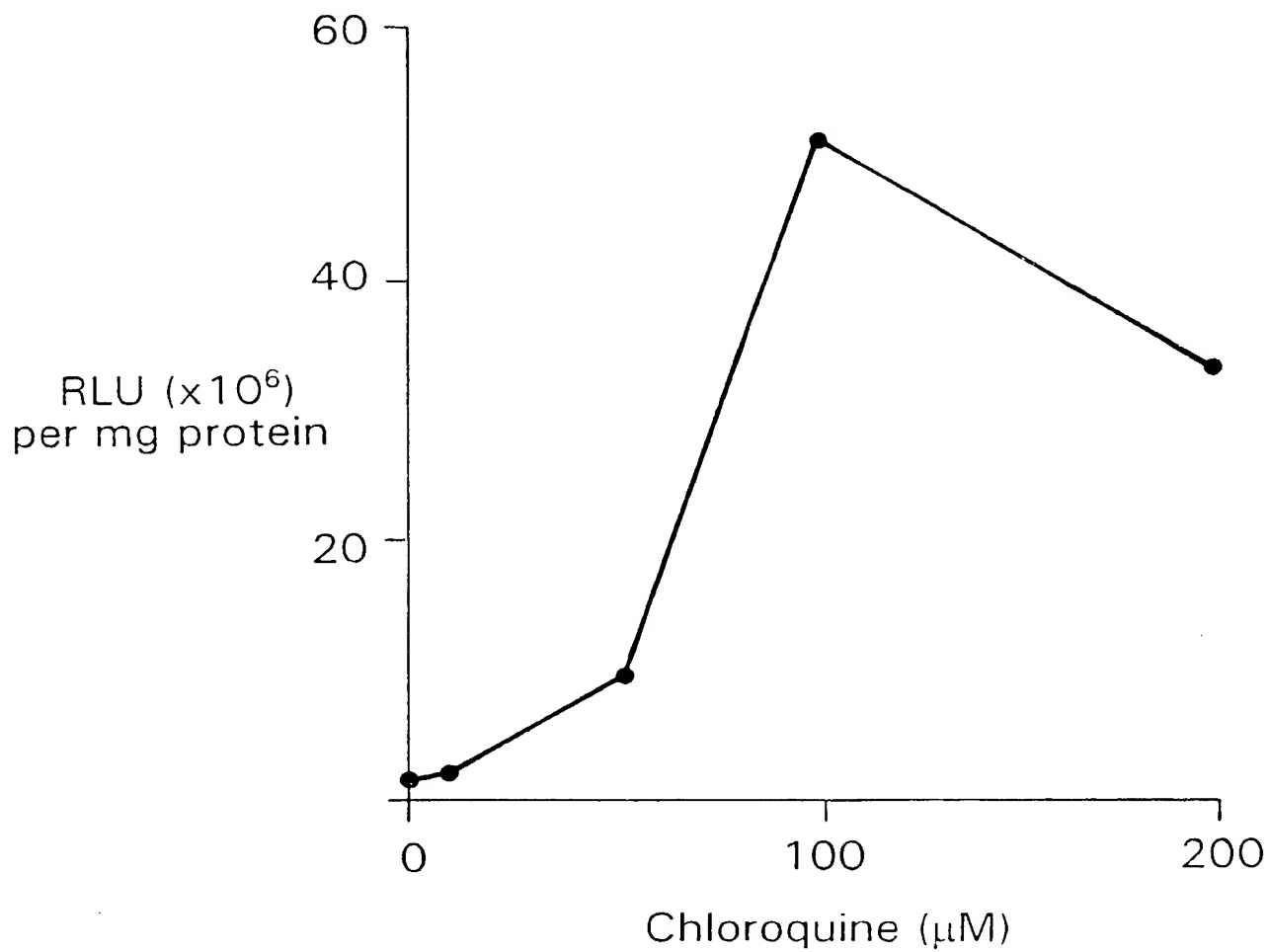


Fig. 1

72. An airway epithelial cell transfected with a complex comprising an isolated nucleic acid and a glycosylated polylysine and at least one of chloroquine, glycerol and a fusogenic peptide.

5                    73. The airway epithelial cell of claim 72, wherein said isolated nucleic acid is DNA encoding CFTR and said glycosylated polylysine is lactosylated polylysine.

10                   74. A composition for transfection of airway epithelial cells comprising a complex comprising an isolated nucleic acid and a lactosylated polylysine, wherein said isolated nucleic acid is DNA selected from the group consisting of DNA encoding an asthma gene, DNA encoding  $\alpha$ 1AT, a gene affecting idiopathic pulmonary fibrosis, DNA encoding SP-B and DNA encoding SP-C.

15

75. The composition of claim 74, further comprising at least one of chloroquine, glycerol and a fusogenic peptide.

20                   76. The composition of claim 74, wherein said airway epithelial cells are transfected *in vitro*.

77. The composition of claim 74, wherein said airway epithelial cells are transfected *in vivo*.

25

63. An *in vitro* cell transfection kit comprising a selection of glycosylated polylysines and instructions for using said kit.

5

64. The kit of claim 63, further comprising a reporter DNA.

65. The kit of claim 63, further comprising at least one of chloroquine, glycerol and a fusogenic peptide.

10

66. The kit of claim 63, said glycosylated polylysine having a sugar component selected from the group consisting of lactose,  $\alpha$ -glucose,  $\beta$ -galactose, mannose, mannose-6-phosphate, fucose and N-acetylglucosamine.

15

67. The kit of claim 64, wherein said reporter DNA is selected from the group consisting of a chloramphenicol acetyl transferase gene, a luciferase gene, a green fluorescent protein gene, and a  $\beta$ -galactosidase gene.

68. A nebulizer having a composition comprising a complex comprising an isolated nucleic acid and a glycosylated polylysine placed therein.

20

69. The nebulizer of claim 68, wherein said isolated nucleic acid is DNA encoding CFTR and said glycosylated polylysine is lactosylated polylysine.

70. A bronchoscope having a composition comprising a complex comprising an isolated nucleic acid and a glycosylated polylysine placed therein.

25

71. The bronchoscope of claim 70, wherein said isolated nucleic acid is DNA encoding CFTR and said glycosylated polylysine is lactosylated polylysine.

56. The method of claim 51, said glycosylated polylysine having a sugar component selected from the group consisting of lactose,  $\alpha$ -glucose,  $\beta$ -galactose, mannose, mannose-6-phosphate, fucose and N-acetylglucosamine.

5 57. The method of claim 56, wherein said glycosylated polylysine comprises lactosylated polylysine.

58. The method of claim 57, wherein about 10% to about 60% of the amino groups of said polylysine have a lactose molecule substituted thereon.

10

59. The method of claim 57, wherein the weight to weight ratio of lactosylated polylysine to DNA in said complex is about one to one to about fifteen to one.

15

60. The method of claim 59, wherein the weight to weight ratio of lactosylated polylysine to DNA in said complex is about three to one to about nine to one.

20 61. A method of identifying a test compound capable of modulating the activity of CFTR comprising

transfecting airway epithelial cells in the presence or absence of said test compound with a complex comprising an isolated nucleic acid encoding CFTR, or a biologically active fragment thereof, and a glycosylated polylysine, and

25 measuring the activity of CFTR in said cells, wherein a higher or a lower level of CFTR activity in the presence of said test compound compared with CFTR activity in cells in the absence of said test compound is an indication that said test compound is capable of modulating the activity of CFTR.

30 62. A compound identified according to the method of claim 61.

48. The lactosylated polylysine nucleic acid complex of claim 47, wherein the weight to weight ratio of lactosylated polylysine to said isolated nucleic acid in said complex is about nine to one.

5 49. A kit comprising an isolated nucleic acid encoding CFTR, or a biologically active fragment thereof, a glycosylated polylysine and instructions for using said kit for transfection of airway epithelial cells.

10 50. A kit comprising an isolated nucleic acid encoding CFTR, or a biologically active fragment thereof, a glycosylated polylysine and instructions for using said kit for treatment of cystic fibrosis in a human patient.

15 51. A method of treating a human patient having cystic fibrosis, said method comprising administering to said human a pharmaceutical composition comprising a complex comprising an isolated nucleic acid encoding CFTR, or a biologically active fragment thereof, and a glycosylated polylysine.

20 52. The method of claim 51, said composition further comprising at least one of chloroquine, glycerol and a fusogenic peptide.

53. The method of claim 51, wherein said pharmaceutical composition is administered to said human by a means selected from the group consisting of aerosol nebulizer, bronchoscopy and injection *in utero*.

25 54. The method of claim 51, wherein said isolated nucleic acid comprises DNA.

30 55. The method of claim 51, wherein said isolated nucleic acid comprises cDNA.

41. The pharmaceutical composition of claim 37, further comprising at least one of chloroquine, glycerol and a fusogenic peptide.

5 42. The pharmaceutical composition of claim 37, said glycosylated polylysine having a sugar component selected from the group consisting of lactose,  $\alpha$ -glucose,  $\beta$ -galactose, mannose, mannose-6-phosphate, fucose and N-acetylglucosamine.

10 43. The pharmaceutical composition of claim 42, wherein said glycosylated polylysine comprises lactosylated polylysine.

15 44. The pharmaceutical composition of claim 43, wherein about 10% to about 60% of the amino groups of said polylysine have a lactose molecule substituted thereon.

45. The pharmaceutical composition of claim 44, wherein about 12% to about 40% of the amino groups of said polylysine have a lactose molecule substituted thereon.

20 46. The pharmaceutical composition of claim 43, wherein the weight to weight ratio of lactosylated polylysine to DNA in said complex is about one to one to about fifteen to one.

25 47. A lactosylated polylysine nucleic acid complex comprising DNA encoding CFTR, or a biologically active fragment thereof, and lactosylated polylysine, wherein about 10% to about 60% of the amino groups of said polylysine have a lactose molecule substituted thereon, and the weight to weight ratio of lactosylated polylysine to said DNA in said complex is about one to one to about fifteen to one, said complex being capable of transfecting airway epithelial cells  
30 when added thereto.

33. The pharmaceutical composition of claim 32, wherein said glycosylated polylysine comprises lactosylated polylysine.

5 34. The pharmaceutical composition of claim 33, wherein about 10% to about 60% of the amino groups of said polylysine have a lactose molecule substituted thereon.

10 35. The pharmaceutical composition of claim 34, wherein about 12% to about 40% of the amino groups of said polylysine have a lactose molecule substituted thereon.

15 36. The pharmaceutical composition of claim 33, wherein the weight to weight ratio of lactosylated polylysine to DNA in said complex is about one to one to about fifteen to one.

20 37. A pharmaceutical composition for treatment of a respiratory disease in a human comprising a complex comprising an isolated nucleic acid and a glycosylated polylysine, wherein said isolated nucleic acid comprises antisense DNA capable of inhibiting the expression of a gene, which gene is required for the development of a respiratory disease in a mammal.

25 38. The pharmaceutical composition of claim 37, wherein said gene is selected from the group consisting of an interleukin gene and a gene affecting leukotriene synthesis.

39. The pharmaceutical composition of 37, wherein said respiratory disease is asthma and said gene is a gene encoding IL-4.

30 40. The pharmaceutical composition of claim 37, wherein said respiratory disease is asthma and said gene is a gene encoding IL-5.



26. The method of claim 23, wherein said respiratory disease is asthma and said gene is a gene encoding IL-5.

5 27. A pharmaceutical composition for treatment of a respiratory disease in a human, comprising a complex comprising an isolated nucleic acid encoding a protein, or a biologically active fragment thereof, and a glycosylated polylysine, wherein said isolated nucleic acid is DNA selected from the group consisting of DNA encoding CFTR, an asthma gene, DNA encoding  $\alpha$ 1AT, a gene affecting idiopathic pulmonary fibrosis, DNA encoding SP-B and DNA encoding  
10 SP-C, said complex being suspended in a pharmaceutically acceptable carrier, said complex being capable of transfecting airway epithelial cells when added thereto.

28. The pharmaceutical composition of claim 27, further comprising  
15 at least one of chloroquine, glycerol and a fusogenic peptide.

29. The pharmaceutical composition of claim 27, wherein said  
respiratory disease is selected from the group consisting of cystic fibrosis, asthma, emphysema, idiopathic pulmonary fibrosis and congenital deficiency of surfactant  
20 protein.

30. The pharmaceutical composition of claim 27, wherein said DNA  
comprises cDNA.

31. The pharmaceutical composition of claim 30, wherein said  
25 cDNA encodes CFTR.

32. The pharmaceutical composition of claim 27, said glycosylated  
polylysine having a sugar component selected from the group consisting of lactose,  $\alpha$ -glucose,  $\beta$ -galactose, mannose, mannose-6-phosphate, fucose and N-  
30 acetylglucosamine.

18. The method of claim 14, wherein said isolated nucleic acid is DNA.

19. The method of claim 18, wherein said DNA is cDNA.

5

20. The method of claim 18, wherein said DNA is selected from the group consisting of DNA encoding CFTR, an asthma gene, DNA encoding  $\alpha 1$ AT, a gene affecting idiopathic pulmonary fibrosis, DNA encoding SP-B and DNA encoding SP-C.

10

21. The method of claim 20, wherein said DNA encodes CFTR.

22. A method of transfecting airway epithelial cells in vivo, said method comprising generating a composition comprising a complex comprising an isolated nucleic acid and a glycosylated polylysine, and adding said complex to said airway epithelial cells.

15

23. A method of transfecting airway epithelial cells comprising generating a composition comprising a complex comprising an isolated nucleic acid and a glycosylated polylysine, wherein said isolated nucleic acid is antisense DNA capable of inhibiting the expression of a gene, which gene is required for the development of a respiratory disease in a mammal, and adding said complex to said cells.

20

24. The method of claim 23, wherein said gene is selected from the group consisting of an interleukin gene and a gene affecting leukotriene synthesis.

25

25. The method of claim 23, wherein said respiratory disease is asthma and said gene is a gene encoding IL-4.

30

10. A method of transfecting airway epithelial cells comprising adding to said cells a composition comprising a complex comprising an isolated nucleic acid and a glycosylated polylysine, wherein said isolated nucleic acid is antisense DNA capable of inhibiting the expression of a gene, which gene is  
5 required for the development of a respiratory disease in a mammal.

11. The method of claim 10, wherein said gene is selected from the group consisting of an interleukin gene and a gene affecting leukotriene synthesis.

10 12. The method of claim 11, wherein said respiratory disease is asthma and said gene is a gene encoding IL-4.

13. The method of claim 11, wherein said respiratory disease is asthma and said gene is a gene encoding IL-5.  
15

14. A method of transfecting airway epithelial cells, said method comprising generating a composition comprising a complex comprising an isolated nucleic acid and a glycosylated polylysine and at least one of chloroquine, glycerol and a fusogenic peptide, and adding said composition to said airway epithelial cells.  
20

15. The method of claim 14, said glycosylated polylysine having a sugar component selected from the group consisting of lactose,  $\alpha$ -glucose,  $\beta$ -galactose, mannose, mannose-6-phosphate, fucose and N-acetylglucosamine.

25 16. The method of claim 15, wherein said glycosylated polylysine is lactosylated polylysine.

17. The method of claim 14, wherein said cells are transfected *in vitro*.  
30

What is claimed is:

1: A method of transfecting airway epithelial cells comprising  
adding to said cells a composition comprising a complex comprising an isolated  
5 nucleic acid and a glycosylated polylysine and at least one of chloroquine, glycerol  
and a fusogenic peptide.

2. The method of claim 1, said glycosylated polylysine having a  
sugar component selected from the group consisting of lactose,  $\alpha$ -glucose,  $\beta$ -  
10 galactose, mannose, mannose-6-phosphate, fucose and N-acetylglucosamine.

3. The method of claim 2, wherein said glycosylated polylysine is  
lactosylated polylysine.

15 4. The method of claim 1, wherein said cells are transfected *in vitro*.

5. The method of claim 1, wherein said isolated nucleic acid is  
DNA.

20 6. The method of claim 5, wherein said DNA is cDNA.

7. The method of claim 5, wherein said DNA is selected from the  
group consisting of DNA encoding CFTR, an asthma gene, DNA encoding  $\alpha$ 1AT, a  
gene affecting idiopathic pulmonary fibrosis, DNA encoding SP-B and DNA  
25 encoding SP-C.

8. The method of claim 7, wherein said DNA encodes CFTR.

9. A method of transfecting airway epithelial cells *in vivo*  
30 comprising adding to said cells a composition comprising a complex comprising an  
isolated nucleic acid and a glycosylated polylysine

### Assessment of *In Vivo* Correction of the CF Defect in Animal

#### Models

5 The tracheal xenograft model may be used for these studies. To assess correction of the CF defect *in vivo*, immune incompetent mice which have been given a denuded rat-trachea transplant under the skin on which CF airway epithelial cells are grafted are used. Aliquots of 100  $\mu$ l of plasmid/polylysine complex plus 50  $\mu$ M chloroquine are administered to the animals at the site of the xenograft. After about 4 hours, the mixture is removed and correction of the CF-associated transepithelial potential difference with amiloride stimulation is  
10 measured after 36-40 hours post transfection.

Alternatively, any of the other animals discussed herein may also be used for administration of CFTR.

As discussed herein, the invention should also be construed to include treatment of CF *in utero* using lactose substituted polylysine DNA  
15 complexes.

The disclosures of each and every patent, patent application and publication cited herein are hereby incorporated herein by reference in their entirety.

20 While this invention has been disclosed with reference to specific embodiments, it is apparent that other embodiments and variations of this invention may be devised by others skilled in the art without departing from the true spirit and scope of the invention. The appended claims are intended to be construed to include all such embodiments and equivalent variations.

## Transfer of Reporter Genes into Airway Epithelial Cells in

### Animals

Adult C57/BL6 mice provide an effective animal model for testing the efficacy of glycosylated polylysine vehicles for gene transfer into respiratory epithelial cells *in vivo*. Each of the glycosylated polylysines described herein may be examined in this model for the ability to efficiently transfer DNA into airway epithelial cells *in vivo* as follows.

The glycosylated polylysine is combined with the reporter plasmid CMV-LacZ (CAYLA or other supplier) as described. Aliquots of 100  $\mu$ l containing 2-10  $\mu$ g of plasmid are administered to anesthetized mice via the intranasal or intratracheal route. This method is successful when adenoviral vectors are used and results in reliable gene expression in distal airway and parenchymal cells. One, three, or six doses are administered to the animals intranasally on sequential days. The animals are sacrificed two days following the last dose and are processed for histopathology and  $\beta$ -galactosidase expression. Controls may include recombinant adenoviral vectors (AdCMVLacZ/sub360) to assess cell specificity and distribution of transgene expression (St. George *et al.*, 1995, "Efficacy of Adenoviral Vectors in Airway Epithelium" Cystic Fibrosis Conference, Abstract #151). Newborn lung and fetal lungs from animals may also be tested using the methods described herein.

### Transfer of the CFTR Gene into Animals.

CFTR-encoding plasmids are complexed with glycosylated polylysine and are administered to an animal following the procedures just described for transfer of reporter plasmids to animal airway epithelial cells. Expression of CFTR is detected as described herein. Administration of lactose substituted polylysine to the airway cells of animals may be accomplished by aerosol through the nasal passages, by bronchoscopy or by any other method available in the art, such as by tracheal catheter.

In addition to mice, rabbits and other vertebrate animals may be used, including non-human primate animals, to examine the introduction of CFTR into the airway epithelial cells of these animals using the methods described herein.

(A) Measurement of  $^{125}\text{I}$ -efflux from CF/T43 cells (Figure 15).  $^{125}\text{I}$ -efflux from cultured CF cells is a measure of whether the CFTR defect in cells has been corrected, wherein correction of the defect results in an increase in efflux of  $^{125}\text{I}$ .  $^{125}\text{I}$ -efflux is measured in a manner similar to that described by Marks *et al.* (1988, *10th International CF Congress*, Sydney, Australia). Essentially, immortalized CF airway epithelial cells (CF/T43) and primary CF and non-CF airway epithelial cells are transfected with the lactosylated polylysine/CFTR plasmid complex using the transfection conditions described herein. The cells are seeded in 35 mm dishes at a cell concentration of  $10^6$  cells per dish. Chloride channel activity in the presence of additives such as forskolin, is assayed by measuring the rate of  $^{125}\text{I}$ -efflux (Venglarik *et al.*, 1990, *Am. J. Physiol.* **259**:C358-C364; Santos *et al.*, 1994, *Biochim. Biophys. Acta* **1195**:96-102; Drumm *et al.*, 1990, *Cell* **62**:1227-1233).

(B) Measurement of SPQ fluorescence. Immortalized CF airway epithelial cells (CF/T43) and primary CF and non-CF airway epithelial cells are cultured on glass coverslips and are transfected with the lactosylated polylysine/CFTR plasmid complex as described herein. Following transfection, the cells are further incubated for 36-48 hours. The cells are then washed with PBS and the halide sensitive fluorophore 6-methoxy-N-(3-sulphopropyl)quinolinium (SPQ) is added. The cells are analyzed according to the method of Yang *et al.* (1993, *Hum. Mol. Gen.* **8**:1253-1261). SPQ fluorescence is quantitated and any increase in fluorescence is an indication that the CFTR defect in the cells has been corrected.

The results of experiments designed to measure CFTR activity by assessing the  $\text{Cl}^-$  activity of the protein by  $^{125}\text{I}$  efflux in the presence of the described stimulants, are presented in Figure 15. In this experiment, T-84 cells expressing cells were examined and exhibited a  $\text{Cl}^-$  efflux rate per minute of 3-4 (Figure 15, Panel A). CF/T1 cells whose defect was corrected by the addition of wild type CFTR, also exhibited an efflux rate which was similar to the response of T-84 cells (Figure 15, Panel B). As expected, CF/T43 cells exhibited no response to the stimulation mixture.

Several other immortalized cell lines were examined to determine the effect of the potentiating agents in increasing the efficacy of transfection of cells. The cells used comprised BEAS2B cells which are immortalized epithelial cells obtained from a non-CF patient (Reddell et al., 1988, Cancer Res. 48:1904-1909). CF/T1 (wild type) immortalized airway cells were also used. These cells were obtained from a CF patient having the  $\Delta F508$  mutation which had been previously transfected with wild type CFTR (Olsen et al., 1992, Humam Gene Therapy 3:253-266). Both of these cells were transfected as described herein in the presence or absence of various additives as indicated in Figure 18. In each case, the cells responded to the additives in a similar manner wherein 50 or 100  $\mu M$  of chloroquine and 5% glycerol yielded the greatest increase in transfection efficiency as measured by expression of luciferase in transfected cells.

The data presented herein establish that the invention can be construed to include transfection mixtures having additional compounds added thereto, which compounds serve to enhance the transfection efficiency of the desired cells. Glycerol or a fusogenic peptide may be added to the transfection mixture, either before, concurrently or following addition of the DNA to the cells.

#### **Examination of the Efficiency of Gene Expression**

In experiments designed to examine the efficiency of gene expression, the reporter gene, pCMVLacZ complexed to lactosylated polylysine was used. Cells were transfected with the reporter gene and expression of the gene was assessed by staining the cells with X-gal. The results of a typical experiment of this type are presented in Figure 16. Approximately 40% of the cells exhibited intense blue staining. After three subsequent transfections, 90% of the cells exhibited moderate to intense blue staining which was not evident in control untransfected cells. These experiments establish that CF/T43 cells may be transfected with high efficiency using the composition of the invention as described herein.

#### **Assessment of Whether Transfection of the *CFTR* Gene Results in Functional Correction of the CF Defect in Airway Epithelial Cells**



polylysine should serve to enhance transfection of airway epithelial cells in a safe and effective manner.

The amount of chloroquine to be included in the transfection mixture for administration to humans or animals may vary from about 25  $\mu$ M to about 200  $\mu$ M per dose. Preferably, the amount of chloroquine to be included in the mixture is from about 50  $\mu$ M to about 100  $\mu$ M.

Other enhancing agents may be used in the transfection mixture in place of or in addition to chloroquine. Such agents include, but are not limited to, bioactive peptides and glycerol. Suitable peptides include, but are not limited to, E5CA-GLFEAIAEFIEGGWEGLIEGCA (Midoux *et al.*, 1993, *Nucleic Acids Res.* **21**:871-878), HA-2-GLFEAIAAGFIENGWEGMIDGGGC (Wagner *et al.*, 1992, *Proc. Natl. Acad. Sci. USA* **89**:7934-7938) and JTS-1-GLFEALLELLESLWELLLEA (Gottshalk *et al.*, 1996, *Gene Ther.* **3**:448-457).

Examples of the enhancement of transfection by glycerol and the fusogenic peptide E5CA are now described.

Cells were transfected with pCMVLuc complexed to lactosylated polylysine in the presence or absence of glycerol and/or chloroquine. Following a four hour incubation period with the transfection mixture, the cells were treated as described herein and were incubated for a further 48 hours. The cells were then lysed and luciferase activity expressed therein was assessed as described herein. It is evident from the data shown in Figures 10 and 11 that glycerol enhances transfection of both CF/T43 cells and primary airway epithelial cells when the cells are transfected either with or without chloroquine.

In a similar manner to that just described, CF/T43 cells were transfected for three hours with a reporter gene complexed to gluconoylated polylysine in the presence of increasing amounts of the fusogenic peptide, E5CA. The results of these experiments are shown in Figure 12. It is evident that the transfection efficiency of the cells was markedly enhanced in the presence of fusogenic peptide. The enhancement of luciferase gene expression in CF/T43 cells transfected with a lactosylated polylysine DNA complex is summarized in Figure 13.

expression of *CFTR* in transfected cells may be assessed by measuring the amount of *CFTR* protein in the cells using any one of the several methods as described herein.

Western blotting analysis may be performed using antibody to the R domain of *CFTR* as described in Wei *et al.* (1996, *J. Cell. Physiol.* **168**:373-384) and available technology described for example in Sambrook *et al.* (Molecular Cloning: A Laboratory Manual, Cold Spring Harbor, NY, 1989).

Antibodies are prepared by synthesizing peptides from the deduced amino acid sequence of the R domain and the first ATP binding fold. Peptides may be obtained preferably from regions which are highly conserved between the bovine and human *CFTR* sequences (Diamond *et al.*, 1991, *J. Biol. Chem.* **266**:22761-22769).

To obtain antibody directed against the R domain of *CFTR*, peptides including that domain are covalently coupled to tuberculin purified protein derivative (PPD) and are then inoculated into rabbits. Antibody is then purified from serum obtained from the rabbits at periodic intervals. The technology for making antibodies directed against specific peptides is well known in the art and is described for example, in Harlow *et al.* (1988, *In: Antibodies, A Laboratory Manual*, Cold Spring Harbor, NY). Antibodies to the C terminus domain of *CFTR* may also be used (Mulberg *et al.*, 1994, *Neuroreport* **5**:1684-1688).

Alternatively, expression of *CFTR* may be assessed using a plasmid containing *CFTR* having a M2-901 epitope tag (Howard *et al.*, 1995, *Am J. Physiol.* **269**:1565-1576). Protein expressed by this plasmid may be detected using M2 antibody which is commercially available (Eastman Kodak Co.) and the methods described by Howard *et al.* (*supra*).

The invention further includes administration of chloroquine or other enhancing agent in combination with lactose substituted polylysine *CFTR* DNA complexes to patients having CF. Administration of chloroquine to humans is known for the prevention of malaria infection. As described herein, chloroquine enhances DNA transfer of sugar substituted polylysine DNA complexes into cells. Thus, administration of this compound in combination with DNA and glycosylated

about 90%. The highest transfection efficiency in these cells was accomplished using 5% glycerol and 10 µg of fusogenic peptide in the transfection medium. As shown in Table 1, other combinations of potentiating agents were also used to successfully transfect these cells.

5

**TABLE 1**

In situ hybridization of CF cells in primary culture to detect CFTR mRNA after transfection with pAdCFTR complexed to lactosylated polylysine

<u>Cells<sup>a</sup></u>	<u>CFTR Expressing</u>		
	<u>Additives</u>	<u>High</u>	<u>Medium</u> <u>Transfected</u> <u>Percentage of total</u>
<u>counted</u>			
None		22	58 80
glycerol + Fusogenic peptide <sup>b</sup>		67	22 89
Glycerol		44	56 100
Chloroquine + Fusogenic peptide		31	68 99
Chloroquine		23	66 89
Plasmid		0	10 --
<u>Three Additions</u>			
Glycerol + Fusogenic peptide <sup>b</sup>		30	65 95
Glycerol <sup>b</sup>		35	54 94
Chloroquine + Fusogenic peptide		34	62 91

<sup>a</sup> Based on the number of intensely blue (high) or moderately blue (medium) cells.

<sup>b</sup> Average of 2 separate experiments which deviated by 10% or less.

30

The plasmid/polylysine complex transfected cells may be added to airway epithelial cells as described herein. In addition to assessing expression of *CFTR* by measuring *CFTR*-specific mRNA using *in situ* hybridization, the

In summary, the data described herein establish that polylysine which is partially substituted with either glycosyl or gluconoyl residues is an effective non-viral vehicle for transfer of DNA into airway epithelial cells.

However, glycosylated polylysine, and in particular lactosylated polylysine, is most effective in mediating gene transfer into airway epithelial cells. The presence of chloroquine or other additives as described herein, in the transfection medium enhanced gene transfer in airway epithelial cells even in primary cell cultures.

Thus, according to the present invention, a novel method of transfer of the CF gene into airway epithelial cells has been discovered, which method is useful for treatment of CF. As noted herein, glycosylated polylysines are largely non-immunogenic; thus, they are superior to other non-viral vehicles and are vastly superior to viral vectors as a gene therapy approach for treatment of CF.

#### **Transfer of the *CFTR* Gene into Primary Cells in Culture Using Lactosylated Polylysine as a Delivery Vector**

The technique of *in situ* hybridization was used to demonstrate transfer of the *CFTR* gene into the immortalized cell line, CF/T43, and into cells in primary culture. An example of the results of this type of experiment is shown in Figure 17. For these studies, cells were grown on coverslips and were transfected with 3  $\mu$ g of the plasmid, pAd*CFTR*, or the plasmid, pBQ*CFTR* (Drumm et al., 1990, Cell **62**:1227-1233) complexed to 9  $\mu$ g of lactosylated polylysine. Cells were transfected on three consecutive days in the presence of 5% glycerol and 10  $\mu$ g fusogenic peptide, in the case of the primary cells, and in the presence of 100  $\mu$ M chloroquine and 5% glycerol, in the case of the immortalized cells. Other combinations of potentiating agents were also used (Table 1). Expression of *CFTR* was detected by *in situ* hybridization using exon 14 of *CFTR* labeled with deoxygenin as a probe. Labeled or unlabeled cells were visualized in a Nikon Diaphot 300 microscope and the amount of label was quantitated therein.

A variety of control cells, including plasmid which was not complexed with lactosylated polylysine, exhibited about a 25% transfection efficiency. In contrast to the control cells, the transfection efficiency of primary cultures of cells obtained from nasal polyps or trachea was between about 70 and

lactosyl polylysine had decreased, likely due to the fragility of these particular cells and their response to the presence of chloroquine in the transfection medium.

The concentration of chloroquine required to induce maximum transfection efficiency in human non-CF airway epithelial cells was observed to be lower than that observed in CF/T43 cells (compare Figures 5C and Figure 8). An optimal level of luciferase expression was observed in primary cells in the presence of 50  $\mu$ M chloroquine.

In summary, the results just described establish that primary cell cultures (both CF and non-CF) may be efficiently transfected with DNA using glycosylated polylysine as a transfection vehicle. Lactosylated polylysine is superior to other sugar substitutions and gluconoylated polylysine is an inefficient vehicle for gene transfer in these cells.

#### **Endogenous Lectins on CF/T43 Cells Which Bind**

##### **Lactose**

To assess whether the observed enhanced transfection efficiency using lactosylated polylysine is related to any direct interaction between lactosylated polylysine and the carbohydrate binding proteins on the outer surface of the cells, binding studies were conducted. In particular, the binding of lactose-neoglycoprotein was examined.

Lactose-neoglycoprotein was prepared by conjugating lactose to BSA-FITC. This compound was added to CF/T43 cells in a binding assay and was observed to bind to the surface of these cells at 4°C (Figure 9A). Since binding was blocked by the addition of 0.1 M or 0.2 M lactose, the observed binding was considered to be a specific rather than a non-specific binding of lactose to the cell surface (Figure 9B). FITC-labeled-BSA did not bind under these conditions. These experiments therefore demonstrate the presence of endogenous lactose-binding lectins on the surface of the airway cells.

In additional experiments, increasing concentrations of lactose added to the transfection medium were observed to effect a decrease in the expression of luciferase. When lactose was added to the transfection medium at a concentration of 0.1 M, a 60 % decrease in luciferase expression was observed.

The presence of chloroquine in the transfection mixture containing lactosylated polylysine also resulted in an overall increase in expression of luciferase (Figure 5C). When chloroquine was absent, luciferase activity was observed to be only 2% of the level observed when 100  $\mu$ M of chloroquine was added to the transfection mixture.

#### **Transfection of Primary CF Airway Epithelial Cells in Culture**

CF airway epithelial cells grown from tracheal tissue explants were transfected and examined for the expression of luciferase using the glycosylated or gluconoylated substituted polylysines as the vehicle and pCMV*Luc* as the expression plasmid. The results of experiments conducted in two different primary cultures are shown in Figure 6. A high level of luciferase gene expression was observed when lactosylated polylysine served as the transfection vehicle. Other glycosylated polylysines resulted in approximately 10-fold less luciferase activity in transfected CF cells, and in the case of Fuc-substituted polylysine, luciferase activity was 80-fold less than that observed using lactosylated polylysine.

#### **The Use of Glycosylated Polylysines as Vehicles for Transfer of DNA into Primary Human (non-CF) Airway Epithelial Cells**

Tissue explants were obtained from tracheal tissue which was itself obtained from lung transplant patients. Airway epithelial cells were grown from the tissue explants and were transfected as described herein using a variety of sugar substituted polylysines. These results are shown in Figure 7.

Consistent with the results described above, lactosylated polylysine was an effective vehicle for transfection of non-CF airway epithelial cells (Figure 7A).  $\alpha$ -Glucose- or  $\beta$ -galactose-substituted polylysines were also effective vehicles. In another experiment, galactosylated polylysine proved to be less than 50% as effective for transfection compared with lactosylated polylysine (Figure 7B). Non-substituted polylysine was only 8% as effective as lactosylated polylysine.

When the time of transfection was increased from 4 to 6 hours, the expression of luciferase was increased by 40% when lactosylated polylysine was used as the transfection vehicle. However, by 8 hours, the transfection efficiency of

expression when used to transfect cells. The levels of luciferase activity ranged from 3.9 to 0.3 X 10<sup>8</sup> RLU/mg protein in the following order of polylysine complexes used in the transfection mixtures:  $\beta$ -Gal =  $\alpha$ -Glc = Lac >  $\alpha$ -Gal = Rha = Man >  $\beta$ -GalNAc > GlcA >  $\alpha$ -GalNAc =  $\alpha$ -Fuc.

5                    In separate experiments, GlcNAc-substituted polylysine was not as efficient a vehicle as lactosylated polylysine; GlcNAc-substituted polylysine transfected cells exhibited less than 50% of the luciferase activity observed in cells transfected with lactosylated polylysine. Further, non-substituted polylysine was only 10% as effective as lactosylated polylysine in effecting expression of  
10                    luciferase in transfected cells. Thus,  $\alpha$ -glucose,  $\beta$ -galactose and lactose substituted polylysine are superior to other sugar substitutions on polylysine for transfer of DNA into immortalized airway epithelial cells.

#### **Optimal Conditions for Lactosylated Polylysine Mediated Gene Transfer**

15                    The data presented in Figure 4 establish that lactosylated polylysine is a highly efficient vehicle for the transfection of CF/T43 cells. To determine the optimal conditions for use of lactosylated polylysine as a transfection vehicle in CF/T43 cells, the following experiments were conducted.

20                    The ratio (w/w) of the lactosylated polylysine to pCMV*Luc* was varied to determine the optimum ratio for efficient transfection of plasmid. These results are shown in Figure 5A. When a 4:1 ratio was used in the transfection mixture, the expression of luciferase in transfected cells was increased two-fold, compared with a 3:1 ratio.

25                    The effect of time on transfection efficiency was also determined. When the time of transfection was increased to 8 hours from 4 hours, luciferase activity was increased in transfected CF/T43 cells (Figure 5B). However, at a transfection time of 8 hours, some morphological changes were observed in the cells. The optimal time of transfection using lactosylated polylysine in the presence of 100  $\mu$ M chloroquine was approximately 6 hours. In these cells, expression of  
30                    luciferase continued for up to 120 hours.

time post transfection, the cells were harvested and luciferase activity was measured (Figure 3B). Maximal luciferase activity was observed between 48 and 96 hours post transfection. In fact, when the time was extended to 120 hours post transfection, a very high level of luciferase activity (130% of that observed at 48 hours post transfection) was observed. Since during the course of this latter experiment the cells were incubated in growth medium, the cell number actually increased, therefore apparently lowering the amount of luciferase activity when measured as activity per mg of protein.

### The Use of Glycosylated Polylysines for Transfection of CF/T43

#### 10 Cells

Glycosylated polylysines containing varying numbers of mono or disaccharides were prepared as described herein. Polylysine substituted with monosaccharides contained an average of  $77 \pm 10$  sugar residues corresponding to the substitution of  $41 \pm 5\%$  of the amino groups of polylysine (DP-190).

15 Lactosylated polylysine contained an average of 66 lactose residues corresponding to the substitution of 34% of the amino groups.

Glycosylated polylysine/plasmid complexes were made having the lowest polymer to DNA ratio (2 or 2.5  $\mu\text{g}$  polylysine substituted with monosaccharides per  $\mu\text{g}$  pCMV*Luc* and 3  $\mu\text{g}$  lactosylated polylysine per  $\mu\text{g}$  pCMV*Luc*). At these concentrations and ratios the following was observed: (i) a complete retardation of all the DNA during electrophoresis; (ii) complexes which had a pH near 7.0; (iii) a complete association of glycosylated polylysine with the DNA; and iv) high efficiency gene transfer.

When transfection mixtures were prepared in this manner, the level of luciferase activity was observed to vary with the type carbohydrate contained on the polylysine (Figure 4). The use of polylysine substituted with  $\beta$ -Gal,  $\alpha$ -Glc or lactose complexed with pCMV*Luc* yielded luciferase activity at levels of 322%, 298% and 289%, respectively, when compared with the use of gluconoylated polylysine. Polylysine substituted with Rha, Man and  $\alpha$ -Gal also yielded high expression of luciferase activity; however, several other carbohydrate substitutions, namely  $\alpha$ -L-Fuc and  $\alpha$ -GalNAc yielded negligible amounts of luciferase gene



the lowest possible molar ratio between the vehicle and DNA. In other words, the ratio of polylysine to DNA was adjusted so that the transfection medium did not contain any DNA-free vehicle which might otherwise contribute to cellular toxicity (Erbacher *et al.*, 1995, *supra*). When the vehicle:plasmid ratio was 2:1 there were  
5 no observed morphological changes in the cells. When non-substituted polylysine was used as a vehicle complexed with pCMV*Luc* at a ratio of 2:1 (w/w), luciferase was expressed to a level of only 40% of that observed using gluconoylated polylysine.

To assess the effect of cell number on transfection efficiency and  
10 gene expression, the number of CF/T43 cells in each transfection assay was varied while maintaining the plasmid concentration at 1  $\mu$ g and the ratio of gluconoylated polylysine to DNA at 2:1 (w/w). In the majority of the experiments, CF/T43 cells were seeded at a concentration of  $1.5 \times 10^5$  cells per 25 mm well. However, when  
15  $2 \times 10^5$  cells per well were used, a higher level of luciferase activity was observed (Figure 2B). When the cell concentration was increased to greater than  $2 \times 10^5$  cells per well, no additional increase in luciferase activity was observed. Thus, the number of cells seeded in each well has an effect on the transfection efficiency of the cells.

The effect of the length of time of transfection on transfection  
20 efficiency and gene expression was assessed as follows. DNA was added to cells in a standard transfection mixture at a gluconoylated polylysine to DNA ratio of 2:1. Cells were incubated with the DNA transfection mixture for 4 to 10 hours in the presence of 100  $\mu$ M chloroquine. At each time point tested, the transfection  
25 mixture was removed from the cells, the cells were washed and incubated in fresh medium for 48 hours prior to lysis and luciferase assay. From Figure 3A it is evident that the amount of luciferase activity increased about 4-fold in transfected CF/T43 cells when the transfection time was increased from 4 to 10 hours. In another experiment using 5  $\mu$ g of plasmid, luciferase activity was observed to peak a transfection time of 6 hours.

30 To assess the effects of incubation time post transfection on gene expression in transfected cells, cells were transfected for 4 hours, and at various

Controls included cells transfected with either of the plasmids or the vehicles on their own.

### **Optimal conditions for Gene Transfer Using Gluconoylated Polylysine**

5                   A series of experiments were performed to determine the optimum conditions for the expression of the luciferase gene following transfection of a luciferase encoding plasmid into CF/T43 cells.

                  To assess the effects of chloroquine on transfection, cells were transfected in the presence of increasing concentrations of this compound. The use  
10               of chloroquine in the transfection mixture resulted in an increase in the transfection efficiency of CF/T43 cells by the gluconoylated polylysine/plasmid complex in a concentration-dependent manner (Figure 1). The amount of luciferase activity expressed in cells transfected with a chloroquine-containing mixture was 75-fold higher than in cells transfected in the absence of chloroquine. An approximately  
15               26-fold increase in luciferase activity was observed when 100  $\mu$ M chloroquine was used compared with the level of luciferase activity in cells transfected in the presence of 10  $\mu$ M chloroquine. When 200  $\mu$ M chloroquine was used the level of luciferase activity was significantly decreased in transfected cells. Fetal bovine serum, at a concentration of 1-10%, had little effect on the transfection efficiency  
20               during a 4 hour transfection time period. Thus, the concentration of chloroquine used affected the transfection efficiency and the highest level of reporter gene expression was observed at a chloroquine concentration of 100  $\mu$ M.

                  To assess the effect of plasmid concentration on transfection efficiency and gene expression, the concentration of the plasmid pCMVLuc (pUT  
25               650) was varied from 0.1 to 10  $\mu$ g, while maintaining the gluconoylated polylysine:plasmid ratio at 2:1 (Figure 2A). When the concentration of the plasmid complex was linearly increased, luciferase activity also increased linearly. A concentration of 1  $\mu$ g of pCMVLuc was used in subsequent experiments, unless otherwise stated.

30               Increasing amounts of plasmid did not result in increased toxicity to cells because gluconoylated polylysine/plasmid complexes were designed to have

In experiments designed to measure the efficiency of gene expression, the plasmid pCMV*LacZ* may also be used, wherein gene expression is measured by measuring  $\beta$ -galactosidase activity using the chromogenic reagent X-gal.

#### 5                    **Binding of FITC Neoglycoproteins**

Bovine serum albumin (BSA) was lactosylated and subsequently labeled with fluorescein-conjugated isothiocyanate (FITC) as described (Monsigny *et al.*, 1984, *supra*). CF/T43 cells (100-200 cells) were grown on cover slips for 2 days and after removal of the growth medium the cells were incubated with 20-100  
10     $\mu$ g per ml of either lactose-BSA-FITC or BSA-FITC at 4°C for 30 minutes. In some cases, 0.1 or 0.2 M lactose was added with the neoglycoprotein to block binding. The coverslips were washed three times at 4°C with phosphate buffered saline, pH 7.3, and fixed in methanol for 10 minutes at 4°C. After mounting with SlowFade™-light Antifade Kit (Molecular Probes Inc.), binding was assessed using  
15    a Nikon Diaphot 300 microscope.

#### **The Use of Gluconoylated Polylysine as a Transfection Vehicle in CF/T43 Cells**

To establish some optimum parameters for transfection of cells using glycosylated polylysines, gluconoylated polylysines were initially examined in the  
20    transfection assays described herein. Seventy-four amino groups of polylysine (DP 190) were substituted with gluconoyl residues by acylation with  $\delta$ -gluconolactone. This substitution provided a partially neutralized derivative of polylysine which was highly water-soluble and an efficient vehicle for transfecting various cell lines (Midoux *et al.*, WO 95/30020). Gluconoylated polylysine was an efficient vehicle  
25    for the transfer of several luciferase plasmids into CF/T43 cells, resulting in high levels of gene expression in these cells.

Two different luciferase encoding plasmids, wherein the luciferase was placed under the control of two different promoters (pCMV*Luc* or pSV2*Luc*) were used. Either of these plasmids was complexed to gluconoylated polylysine at  
30    a 2 to 1 (w/w) ratio of polylysine to DNA. The plasmid pCMV*Luc* was 26 times more effective than pSV2*Luc* for the expression of luciferase in CF/T43 cells.

the cells were transfected daily for three days. The use of this protocol permits the use of larger quantities of vector/plasmid and this protocol is therefore useful for *in vivo* administration of DNA when it is necessary to maintain higher constant levels of expressed protein products.

5                   To detect luciferase gene expression in transfected cells, the cells from each well were lysed by the addition of 100  $\mu$ l of cell culture lysis reagent (25 mM Tris, pH 7.8; 2 mM EDTA; 2 mM DTT; 10% glycerol; 1% Triton X-100). The cell lysate was incubated for 15 minutes at ambient temperature and was transferred to Eppendorf tubes for centrifugation.

#### 10                   **Measurement of Luciferase Activity**

Luciferase activity was assessed by measuring luminescence following the method of De Wet *et al.* (1987, *Mol. Cell. Biol.* 7:725-737). The assay mixture (270  $\mu$ M coenzyme A, 470  $\mu$ M luciferin, 530  $\mu$ M ATP, 20 mM tricine, 1.07 mM  $(\text{MgCO}_3)_4 \text{Mg}(\text{OH})_2 \cdot 5\text{H}_2\text{O}$ , 2.67 mM  $\text{MgSO}_4$ , 0.1 mM EDTA, 15 33.3 mM DTT, pH 7.8) was added at a ratio of cell lysate:assay mixture of 1:4, which was determined to be the optimum ratio for luciferase activity. Luminescence was recorded on a Luminat luminometer, LB 9501 (EG&G Berthold Analytical Instruments Inc.) for 5 sec and reported as relative light units (RLU). The luciferin/luciferase assay system was stable for more than 60 sec and duplicate 20 wells were assayed in amounts of 5 to 20  $\mu$ l to assure linearity of the results. Different experiments were performed and each was repeated twice. The standard assay points within experiments have a mean RLU of  $3.7 \times 10^7 \pm 2.8 \times 10^6$  SEM (standard error of the mean) (n=11) and  $1.3 \times 10^8 \pm 2.1 \times 10^7$  SEM (n=8) per mg of protein when gluconoylated or lactosylated polylysine, respectively, served as 25 vehicle. A blank assay containing the cell extract but no plasmid, yielded a reading of 120-200 RLU. Protein concentration was determined using the method of Lowry *et al.*, in cells which were lysed with 0.1 M NaOH or in the individual wells containing cells using the lysis buffer as background (Lowry *et al.*, 1951, *J. Biol. Chem.* 193:265-275). The results are expressed as RLU per mg of protein. One 30 picogram of luciferase is equivalent to 11,000 RLU under these assay conditions.

### Transfection Procedures

The polymer/plasmid complexes were prepared as described (Erbacher *et al.*, 1995, *supra*). Gluconoylated or glycosylated polylysine (20-30  $\mu$ g of polylysine dissolved in 0.3 ml of serum-free DMEM) was added to the reporter plasmids (10  $\mu$ g of DNA dissolved in 0.7 ml of serum-free DMEM) and the mixture was incubated for 30 minutes at ambient temperature unless otherwise specified. To prevent precipitation of the complex, the glycosylated polylysine was added to the plasmid at a rate of 15  $\mu$ l per 30 seconds.

The lowest vehicle to DNA ratio (w/w) which exhibited complete retardation of all the DNA during electrophoresis was used. In that instance, all of the DNA was condensed and no free polymer was detected. The ratios were experimentally determined and ranged from 2 to 3. Next, 1 ml of DMEM containing the polymer/plasmid complex was supplemented with 1% heat inactivated fetal bovine serum and 100  $\mu$ M chloroquine, and this mixture was added to each set of cells after removal of the growth medium from the cells.

After incubation at 37°C for 4 hours unless otherwise specified, the transfection mixture was removed and the cells were further incubated at 37°C in 2.0 ml KGM medium without additives. The cells were examined morphologically both after transfection and prior to lysis. Except when specified, all cells appeared normal. After 48 hours or at other time intervals, the cells were processed and expression of the transfected gene was assessed.

In order to increase the efficiency of transfection, the following protocol was adopted. Lactosylated polylysine (2  $\mu$ g in 30  $\mu$ l of DMEM) was added at a rate of 15  $\mu$ l per 30 to 60 seconds to the appropriate plasmid (1  $\mu$ g of plasmid dissolved in 70  $\mu$ l DMEM). The mixture was allowed to sit for an additional 30 minutes at ambient temperature to form a complex. The complex so formed was supplemented as follows: 100  $\mu$ M chloroquine and either 5% glycerol or 10  $\mu$ g fusogenic peptide in the case of immortalized cells; and 5% glycerol with or without fusogenic peptide in the case of the primary cells. Each final mixture was contained in 1 ml of DMEM without serum and was then added to the cells. Thereafter, the transfection procedures was as described herein, and where noted,

sugar residues bound per polylysine molecule was calculated from the sugar content determined by the resorcinol sulfuric acid micromethod (Monsigny *et al.*, 1988, *Biochimie* 70:1633-1649). The 66 lactose residues which bound to polylysine corresponded to a substitution of 35% of the amino groups of the molecule. The  
5 number of monosaccharide residues ( $77 \pm 10$ ) which bound corresponded to a substitution of  $41 \pm 5\%$  of the amino groups of polylysine.

Modification of the above-described procedure yields a compound having even higher activity when complexed to pCMVLuc (Figure 14). These modifications include beginning with 50% less starting material, mixing for 24  
10 hours at 24°C, and washing the precipitate with isopropanol prior to lyophilization. About 20% of the amino groups on the polylysine are substituted with a glycosyl residue following this procedure.

In addition, glycosylated polylysines may be prepared by reductive complexing of lactose to polylysine with cyanoborohydride as described (Matrinez-  
15 Fong *et al.*, 1994, *Hepatology* 20:1602-1608).

#### Cell Culture

The immortalized airway epithelial cell line, CF/T43 was obtained from a CF patient homozygous for the  $\Delta F508$  mutation. The cells were grown as described (Jetten *et al.*, 1989, *Science* 244:1472-1475) in KGM medium.

20 Primary cultures of cells were prepared from tracheal explants or nasal polyps obtained from CF or non-CF patients at the time of surgery. Tracheal pieces were stripped and gently minced and were then placed in 25 cm<sup>2</sup> flasks coated with 25% fibronectin and incubated in LHC-9 medium at 37°C in an atmosphere containing 5% CO<sub>2</sub>. When epithelial cells grew out from the tissue, the  
25 pieces were removed to a new flask until additional epithelial cells grew out. This procedure was repeated several times. In some cases, the cells were obtained by protease treatment of the tissue as described (Wu *et al.*, 1990, *Am. J. Respir. Cell Mol. Biol.* 3:467-478).

Prior to transfection, cells were seeded at  $1.5 \times 10^5$  cells per 25 mm  
30 well in a 12-well plate (Corning). CF/T43 cells were then incubated for 24 hours at 37°C and the primary cells were incubated from 24-48 hours at 37°C.

CAYLA) was used. Thus, the plasmids pCMV*Luc* and pUT 650 each contain the luciferase gene placed under the control of the human CMV promoter.

### Preparation of Gluconoylated Polylysine

Polylysine, HBr (average molecular weight of 40,000; DP-190) obtained from Bachem Feinchemikalien, Bubendorf, Switzerland, was dissolved in H<sub>2</sub>O (1 g in 200 ml) and passed through an anion exchange column (Dowex 2 X 8, OH<sup>-</sup> form, 20-50 mesh, 35 X 2.5 cm) in order to remove bromide ions (Derrien *et al.*, 1989, *Glycoconjugate J.* 6:241-255). The effluent solution was neutralized with 10% *p*-toluene sulfonic acid in water (a non-cytotoxic compound) and was subsequently lyophilized.

Polylysine was partially substituted with gluconoyl residues as described (Derrien *et al.*, *supra*). Briefly,  $\delta$ -gluconolactone (15 mg; Aldrich Chemical Co.) was added to polylysine *p*-toluene sulfonate salt (50 mg) in 3 ml dimethylsulfoxide in the presence of 37  $\mu$ l diisopropylethylamine (Aldrich Chemical Co.); the concentration was adjusted to 1% with water and the solution was stirred for 24 hours at 20°C. Gluconoylated polylysine was precipitated by adding 10 volumes of isopropanol and the precipitate was collected by centrifugation at 1800 X g for 15 minutes. The pellet was washed with isopropanol, collected again by centrifugation, solubilized in distilled water and was lyophilized. The average number of GlcA residues bound per polylysine molecule was determined by <sup>1</sup>H-NMR spectroscopy (300 MHz, D<sub>2</sub>O) and was found to be 74 residues.

### Preparation of Glycosylated Polylysine Conjugates

Polylysine (DP 190) was partially substituted with sugar residues as described (Midoux *et al.*, 1993, *supra*; Erbacher *et al.*, 1995, *supra*). The 4-isothiocyanatophenyl-derivatives of either  $\beta$ -D-Gal,  $\alpha$ -D-Gal,  $\alpha$ -D-Glc,  $\alpha$ -L-Rha,  $\alpha$ -L-Fuc,  $\alpha$ -D-Man,  $\beta$ -D-GlcNAc,  $\alpha$ -D-GalNAc,  $\beta$ -D-GalNAc or  $\beta$ -D-Lac (Monsigny *et al.*, 1984, *Biol. Cell* 51:187-196) were added to polylysine *p*-toluene sulfonate salt in dimethylsulfoxide in the presence of diisopropylethylamine and the mixture was incubated for 24 hours at 20°C. Glycosylated polylysine was precipitated and processed as described above for gluconoylated polylysine. The average number of

Luciferase activity in these cells was enhanced by 75-fold when the transfection mixture included 100  $\mu$ M chloroquine. Luciferase gene expression persisted at high levels for up to at least 120 hours following transfection.

5 Glycosylated polylysine/pCMV*Luc* complexes were compared with the gluconoylated polylysine/pCMV*Luc* complex in immortalized airway epithelial cells. In some cases, pCMV*LacZ* encoding  $\beta$ -galactosidase was used (Gao *et al.*, 1993, *Human Gene Therapy* 4:17-24). It was found that  $\beta$ -galactose,  $\alpha$ -glucose and lactose-substituted polylysines resulted in 320%, 300% and 290% correspondingly higher levels of expression of the reporter gene luciferase than that expressed from 10 gluconoylated polylysine complexed with the same DNA. The amount of luciferase expressed following transfection with the test polylysines ranged from 35 to 2 ng of luciferase per mg of cell protein in the following order:  $\beta$ -Gal =  $\alpha$ -Glc = Lac >  $\alpha$ -Gal = Rha = Man >  $\beta$ -GalNAc >  $\alpha$ -GalNAc =  $\alpha$ -Fuc. These results establish that the transfection efficiency of the subject cells is sugar dependent. Importantly, 15 when primary cultures of either CF or non-CF airway epithelial cells grown from tracheal tissue explants were used, lactosylated polylysine yielded uniformly high expression of luciferase activity.

#### Materials Used in the Studies Described Herein

20 Lysis and assay buffers for determination of luciferase activity were obtained from Promega Corp. Luciferase was obtained from Boehringer-Mannheim Corp. Chloroquine was obtained from Sigma Chemical Co. The media used for cell culture included KGM obtained from Clonetics Corp., LHC-9 obtained from Biofluids Inc., and DMEM obtained from Hazelton Laboratories. Fetal bovine serum was obtained from Biofluids Inc.

25 Expression plasmids encoding the firefly luminescence gene were pSV2*Luc* (5 kb) (Brasier *et al.*, 1989, *Biotechniques* 7:1116-1123), and pCMV*Luc*, having 6.2 kb (Erbacher *et al.*, 1995, *supra*).

The plasmid pSV2*Luc* comprises the luciferase gene placed under the control of the SV40 early promoter, while pCMV*Luc* comprises the luciferase 30 gene placed under the control of the human CMV promoter. When specified, pUT 650 containing a CMV promoter with *Luc::Sh ble* fusion gene (purchased from



as an example and bearing in mind that the use of the kit is in no way limited to this cell type, the kit may be used as follows. Each glycosylated polylysine derivative is individually complexed to a reporter DNA following the procedures described herein. Aliquots of cells are transfected with each complex and expression of the reporter DNA is assessed at selected times post transfection as a measure of transfection efficiency. The glycosylated polylysine derivative yielding the highest efficiency of transfection of the cells will be evident from the levels of expression of the reporter gene in each of the groups of transfected cells. This particular glycosylated polylysine derivative may then be used for all subsequent transfection experiments involving alveolar macrophages. In this way, it is possible to identify a means for high efficiency transfection of any number of different cell types using the kit of the invention.

The unique features of the cell transfection kit of the invention take advantage of the sugar dependent, receptor mediated endocytosis by chemical conjugation of sugars with polylysine which serves to reduce the positive charge of the polylysine while still permitting effective binding with a desired DNA, all DNA's having an overall negative charge which charge facilitates binding of the DNA to compounds such as polylysine. The effectiveness of the method is augmented by the addition of adding glycerol and other known lysosomotropic agents such as chloroquine and fusogenic peptides.

The invention is further described in detail by reference to the following experimental examples. These examples are provided for purposes of illustration only, and are not intended to be limiting unless otherwise specified. Thus, the invention should in no way be construed as being limited to the following examples, but rather, should be construed to encompass any and all variations which become evident as a result of the teaching provided herein.

The results of the experiments described below are now summarized. When gluconoylated polylysine was used as a vehicle, the reporter plasmid pCMV*Luc*, comprising luciferase coding sequences under the control of the human cytomegalovirus (CMV) promoter/regulatory region, yielded a high level of expression of luciferase when transfected into immortalized CF/T43 cells.

Such compounds include glycerol which may be used at a preferred concentration of about 5%, although the use of glycerol at concentrations other than 5% is also contemplated, for example, glycerol concentrations of about 1% to about 8% are contemplated.

5 In addition, it has been discovered in the present invention that fusogenic peptides may be used to enhance the transfection efficiency of glycosylated polylysine DNA complexes into cells. Examples of fusogenic peptides which may be included in the cell transfection kit are provided in the experimental details section herein, and include, but are not limited to, E5CA-  
10 GLFEAIAEFIEGGWEGLIEGCA (Midoux *et al.*, 1993, *Nucleic Acids Res.* **21**:871-878), HA-2-GLFEAIAAGFIENGWEGMIDGGGC (Wagner *et al.*, 1992, *Proc. Natl. Acad. Sci. USA* **89**:7934-7938) and JTS-1-GLFEALLELLESLWELLLEA (Gottshalk *et al.*, 1996, *Gene Ther.* **3**:448-457). While the peptides may be used in the transfection mixture at a preferred concentration of about 10 µg, other  
15 concentrations of fusogenic peptide may also be used. The concentration of fusogenic peptide useful in the methods of the present invention may vary from about 1 µg to about 50 µg.

In addition, the kit may further include an amount of chloroquine, preferably to be used at a concentration of about 100 µM, although other  
20 concentrations of chloroquine are also contemplated. The concentration of chloroquine which may be used may vary from about 30 µM to about 150 µM.

As described herein, DNA complexed with lactosylated polylysine may be transfected into either immortalized or primary cultures of airway epithelial cells with high efficiency. While not wishing to be bound by any theory, it is  
25 believed that transfection of these cells by lactosylated polylysine DNA complexes occurs via receptor mediated endocytosis. Since many other cell types also have the capacity for receptor mediated endocytosis, a kit comprising a variety of glycosylated polylysine derivatives for complexing to DNA will enable the transfection of a number of different cell types. Use of the kit will also enable the  
30 selection of a glycosylated polylysine derivative which effects high efficiency transfection of a particular cell type.

Taking alveolar macrophages

of other genes into that particular cell type. One example of such a cell transfection kit is Perfect Lipid™.

Thus, according to the invention, a kit is provided which comprises a selection of glycosylated polylysine derivatives and optionally includes at least one type of reporter DNA molecule. The selection of polylysine derivatives includes polylysine substituted with lactose,  $\alpha$ -glucose,  $\beta$ -galactose, mannose, mannose-6-phosphate, fucose and N-acetylglucosamine. The amount of each polylysine derivative to be used for transfection of cells will vary depending on any number of factors including the type of DNA and type of cells to be transfected. Typically, the ratio of polylysine derivative to DNA will be from about 1:1 to about 1:15.

By the term "a selection of polylysine derivatives," as used herein, is meant a combination of polylysine derivatives, each one of which is packaged individually so that they are not mixed together.

Reporter DNAs which can be optionally included in the kit include, but are not limited to plasmids or other forms of DNA comprising genes which encode chloramphenicol acetyl transferase, luciferase, green fluorescent protein gene,  $\beta$ -galactosidase, and the like. Essentially, a reporter DNA includes any DNA encoding a product which is detectable in transfected cells. The reporter DNA also comprises a promoter/regulatory sequence for driving expression of the DNA in a cell in which the DNA is transfected. Such promoter/regulatory sequences include, but are not limited to, constitutive promoter /regulatory sequences, such as, but not limited to the SV40 early promoter, the cytomegalovirus immediate early promoter and the Rous sarcoma virus promoter/enhancer, tissue specific promoter/regulatory sequences and inducible promoter sequences. The type of reporter gene and the promoter sequence to which it is operably linked will depend on the type of cells to be transfected and will be readily apparent to one of skill in the art of cell transfection and gene expression. Typically, the concentration of DNA used in a transfection assay will be about 1  $\mu$ g to about 40  $\mu$ g of plasmid mixed in the desired ratio with the glycosylated polylysine.

Also optionally included in the kit are compounds which further enhance the transfection of glycosylated polylysine DNA complexes into cells.

In the case of cell transfection *in vitro*, the instructions comprise directions on how to mix the desired nucleic acid and polylysine to the appropriate proportions, how to treat cells prior to, during and following addition of the transfection mixture to the cells, and how to assess CFTR activity in CFTR  
5 transfected cells. These instructions simply embody the examples provided herein.

In the case of administration of isolated CFTR nucleic acid complexed with glycosylated polylysine to a human, the kit may also comprise a nebulizer into which the transfection mixture is placed. Thus, in this case, instructions for using the kit comprise directions for mixing the isolated nucleic  
10 acid and glycosylated polylysine, and directions including dosages, as described herein, for administering the complex to a human. Such administration directions may also include instructions as to the amount of transfection mixture to be added to the nebulizer and the manner in which the nebulizer is to be used on the patient. Such instructions and directions will depend on factors such as the age of the  
15 individual and the severity of the disease, but in any event, will be apparent to the artisan skilled in the treatment of CF.

The invention should not be construed to be limited to transfection of cells *in vivo* in an animal for the purpose of treatment of a disease. Rather, the invention should also be construed to include transfection of a variety of cells *in*  
20 *vitro* using glycosylated polylysine as a delivery vehicle. Currently available technology for delivery of DNA to cells *in vitro* is limited with respect to the efficiency with which the DNA is delivered. This is particularly true in the case of cells which are considered to be difficult to transfect, such as monocytes and macrophages, for example. Several commercially available cell transfection kits  
25 include a range of lipid compositions which can be mixed with a reporter DNA for transfection into a variety of cell types. The reporter DNA typically comprises a gene encoding a detectable protein product operably linked to a promoter/enhancer sequence for driving expression of the reporter gene when transfected into cells. The lipid composition which effects the most efficient transfection of reporter DNA  
30 into a given cell type is then selected as the composition of choice for transfection

accomplished by following the procedure described in Sekhon *et al.* (1995, *Nature Med.* 1:1201-1203).

Pharmaceutical compositions suitable for administration of glycosylated polylysine nucleic acid complexes to the airway epithelial cells of an animal *in vivo* include, but are not limited to, any of the compositions described  
5 herein for transfection of cells in culture.

Also encompassed by the invention is a method of identifying a compound capable of modulating the activity of CFTR. For example, cells in culture which are transfected with the CFTR gene may be used to identify a  
10 compound which has an affect on CFTR activity. Thus, cells which are transfected with the CFTR gene provide an *in vitro* system for the identification of compounds which modulate CFTR activity. To practice this aspect of the invention, transfected cells expressing CFTR may be treated with a compound which is predicted to affect CFTR activity, and the affect of the compound on CFTR activity may be assessed  
15 by using any of the procedures described herein. In this manner, compounds having an effect on CFTR activity are identified and can be further tested for their capability as therapeutic agents for treatment of CF, also as described herein.

The manner in which a compound capable of modulating CFTR activity is identified is straightforward and simple to practice one armed with the  
20 teaching described herein. For this reason, the invention should be construed to include any and all compounds which are identified following the methods described herein.

Also included in the invention is a kit comprising an isolated nucleic acid comprising a DNA useful for treating a human, for example, but not limited to  
25 CFTR, or a biologically active fragment thereof, a glycosylated polylysine and instructions for using the kit. Optionally, the kit may include one or more of glycerol, fusogenic peptide or chloroquine. The kit is useful for transfection of airway epithelial cells, useful, for example, for the identification of compounds capable of modulating CFTR activity, and is also useful for treatment of humans  
30 having CF. The instructions for using the kit therefore depend on the procedure for which the kit is to be used.

non-human primates (Simon *et al.*, 1993, *Human Gene Therapy* 4:771-780.

Transgenic animal models may also be useful in the invention.

According to the methods of the invention, glycosylated polylysine complexed to CFTR DNA may be administered to a human having CF in a manner similar to that described for the animal models discussed herein. Essentially, the human is anesthetized unless the DNA is to be administered via an aerosol nebulizer, and the DNA/polylysine complex is administered by bronchoscopy, or by using a tracheal catheter, at doses of about 500  $\mu$ g to about 10 mg of DNA and an appropriate amount of glycosylated polylysine in a volume of about 1 ml to about 100 ml depending on the age and size of the individual and the severity of the disease. Typically, a normal adult will receive about 50 ml of a DNA solution having about 5 mg of CFTR DNA. The administration of a compound to a human by aerosol, bronchoscopy, or tracheal catheter, is well known in the art and is described, for example, in Curiel *et al.* (1996, *Am. J. Respir. Cell Mol. Biol.* 14:1-18). It will be appreciated that the precise method of administration of nucleic acid complexed with glycosylated polylysine to a human will depend on any number of factors including the age of the individual and the severity of the disease. The precise mode of treatment of a human will be apparent to the artisan skilled in the treatment of CF and will be tailored by the artisan to the individual being treated.

The DNA/polylysine complex is administered to the human about once a month or less, or about once every two months, or even about once every three months. The treatment regime to be used will depend on several factors including the age of the individual, the extent of the CF symptoms and the overall health of the individual, the length of time since the onset of symptoms, etc.

The invention should also be construed to include treatment of disease *in utero* using glycosylated polylysine DNA complexes. Again, using CFTR as an example, given the availability of genetic tests capable of identifying a defective CFTR gene, it is now possible to determine whether a fetus *in utero* has a defective CFTR gene. Such defects can be corrected *in utero*, prior to the onset of symptoms following birth, thereby preventing many of the sequelae of CF experienced by children and young adults with this disease. Treatment *in utero* is

*situ* hybridization and, in addition, expression is measured in any CFTR assay such as the assays described herein in the experimental details section.

For transfection of cells *in vivo*, a plasmid or other DNA molecule encoding the desired protein is complexed with glycosylated polylysine and the complex is administered to an animal following the procedures described herein for transfer of reporter plasmids to animal airway epithelial cells. Expression of the protein is assessed as described herein. Administration of lactose substituted polylysine/DNA complex to airway epithelial cells of animals may be accomplished by aerosol through the nasal passages, by bronchoscopy or by any other method available in the art, such as by tracheal catheter.

Using CFTR as an example, but appreciating that the invention is not limited solely to the use of CFTR, procedures for transfection of cells *in vivo* are now described. In addition to mice, rabbits and other vertebrate animals may be used, including non-human primate animals, to examine the introduction of DNA, for example, CFTR into the airway epithelial cells of these animals using the methods described herein.

To test the effectiveness of, for example, CFTR added to airway epithelial cells *in vivo*, the tracheal xenograft model may be used (Engelhard *et al.*, 1992, *J. Clin. Invest.* **90**:2598-2607). This model is described in detail herein in the experimental details section. Essentially, the model comprises an immunodeficient mouse having a denuded rat-trachea transplant positioned under the skin on which CF airway epithelial cells are grafted. CFTR nucleic acid complex with glycosylated polylysine suspended in a suitable transfection mixture is administered to the animals at the site of the xenograft. After a period of time, generally about four hours, the mixture is removed. Correction of the CF-associated defect is then assessed by measuring transepithelial potential difference with amiloride stimulation after about thirty six to about forty hours post transfection.

Alternatively, other animals may also be used for administration of CFTR using glycosylated polylysine. Such animal models include, but are not limited to, adult mice and rabbits (Lisby *et al.*, 1996, *Pediatr. Res.* **39**: 389A) and

The invention should thus be construed to include nucleic acid encoding desired proteins and fragments of nucleic acid encoding desired polypeptides; and, nucleic acids and fragments of nucleic acids which are in the antisense orientation to nucleic acid encoding the desired protein or polypeptide.

5 By the term "transfection" as used herein, is meant the transport of nucleic acid into a cell and the expression of the nucleic acid therein.

The term "expression of a nucleic acid " as used herein means the synthesis of the protein product encoded by the nucleic acid.

A suitable transfection mixture for transfer of nucleic acid into  
10 airway epithelial cells comprises any ordinary transfection mixture available in the art, including, but not limited to, isotonic medium, for example, DMEM, which is preferably serum-free. In addition, other compounds may be added to the transfection mixture for the purpose of improving the stability of the complex and/or improving the transfection efficiency in the desired cells. Such compounds  
15 include, but are not limited to, chloroquine, glycerol and fusogenic peptides. It will be appreciated that the amount of such compounds to be added to the transfection mixture will vary depending on any number of factors, including, but not limited to, the type of compound being used, whether transfection is conducted *in vitro* or *in vivo*, the size of the nucleic acid to be transfected and the amount and relative ratios  
20 of the nucleic acid to substituted polylysine. Examples of the use of such enhancing compounds are included herein in the experimental details section.

For transfection *in vitro*, the nucleic acid encoding the gene to be expressed and glycosylated polylysine are added to cells in the appropriate transfection mixture. The cells are incubated for a period of time, generally about  
25 three to about four hours, and the cells are then washed, growth medium is added and the cells are incubated for an additional about twenty four to about seventy two hours. Expression of the gene is assessed using any number of detection methods for the production of protein, including, but not limited to immunological protein detection methods, such as Western blotting, immunofluorescence, ELISA, and the  
30 like. When the nucleic acid to be transfected is CFTR DNA, expression of CFTR nucleic acid is assessed using any of the aforementioned techniques including *in*



capable of base pairing with each other, then the nucleic acids are considered to be complementary to each other at this position. Thus, two nucleic acids are complementary to each other when a substantial number (at least 50%) of corresponding positions in each of the molecules are occupied by nucleotides which normally base pair with each other (e.g., A:T and G:C nucleotide pairs). As defined  
5 herein, an antisense sequence is complementary to the sequence of a double stranded DNA molecule encoding a protein. It is not necessary that the antisense sequence be complementary solely to the coding portion of the coding strand of the DNA molecule. The antisense sequence may be complementary to regulatory  
10 sequences specified on the coding strand of a DNA molecule encoding a protein, which regulatory sequences control expression of the coding sequences.

The antisense nucleic acids which are useful in the present invention include those which have been modified to enhance their stability or otherwise enhance their ability to inhibit gene expression. Antisense nucleic acids which  
15 contain at least one phosphorothioate modification are known to confer upon the oligonucleotide enhanced resistance to nucleases. Specific examples of modified oligonucleotides include those which contain phosphorothioate, phosphotriester, methyl phosphonate, short chain alkyl or cycloalkyl intersugar linkages, or short chain heteroatomic or heterocyclic intersugar ("backbone") linkages. In addition,  
20 oligonucleotides having morpholino backbone structures (U.S. Patent No: 5,034,506) or polyamide backbone structures (Nielsen et al., 1991, Science 254: 1497) may also be used. The examples of antisense oligonucleotide modifications described herein are not exhaustive and it is understood that the invention includes additional modifications of the oligonucleotides of the invention which  
25 modifications serve to enhance the therapeutic properties of the oligonucleotide without appreciable alteration of the basic sequence of the oligonucleotide.

Methods of preparing the oligonucleotides used in accordance with this invention are routine in the art, for example, solid phase synthesis is a well known technique commonly used to synthesize such oligonucleotides. It is also  
30 well known to use similar techniques to prepare other oligonucleotides such as phosphorothioate and alkylated derivatives.

halogens in cells, wherein an increase in efflux of the halogen in cells treated with the putative CFTR gene fragment, which cells are also stimulated by either forskolin or cyclic AMP, identifies the fragment as a biologically active fragment of the CFTR gene.

5                   Also contemplated in the invention is the transfection of cells either *in vivo* or *in vitro* with a DNA molecule which is in the antisense orientation with respect to the coding strand of double stranded DNA. Thus, the invention also includes an isolated nucleic acid having a nucleotide sequence which is in the antisense orientation (*i.e.*, is complementary) to a portion or all of a nucleic acid  
10 encoding a gene, the expression of which is detrimental to a host or cell.

                  During the development of certain respiratory diseases in a human, genes are expressed, which if inhibited, would serve to arrest the development of the disease. Such genes are referred to herein as genes which are required for the development of the respiratory disease. For example, during the development of  
15 asthma in a human, cytokines such as IL-4 and IL-5, and leukotrienes are expressed which facilitate the development of an asthma attack. Inhibition of expression of the genes encoding these products would serve to arrest the development of the asthma attack. The invention therefore contemplates the use of DNA molecules which are in the antisense orientation to genes encoding products which are  
20 required for the development of a respiratory disease.

                  By "complementary" to all or a portion of a gene, as used herein, is meant a sequence of nucleic acid which does not encode the protein specified by the gene. Rather, the sequence which is being expressed in the cells is identical to the non-coding strand of the subject gene and thus, does not encode the protein.

25                   The terms "complementary" and "antisense" as used herein, are not entirely synonymous. "Antisense" refers particularly to the nucleic acid sequence of the non-coding strand of a double stranded DNA molecule encoding a protein, or to a sequence which is substantially homologous to the non-coding strand.

                  "Complementary" as used herein refers to the broad concept of subunit sequence  
30 complementarity between two nucleic acids, *e.g.*, two DNA molecules. When a nucleotide position in both of the molecules is occupied by nucleotides normally

By the term "substantially homologous" as used herein, is meant DNA or RNA which is about 50% homologous, more preferably about 70% homologous, even more preferably about 80% homologous and most preferably about 90% homologous to the desired nucleic acid. Genes which are homologous to the desired gene should be construed to be included in the invention provided they encode a protein or polypeptide having a biological activity substantially similar to that of the desired gene.

The desired nucleic acid useful in the invention is nucleic acid encoding a gene which when administered to a mammal, preferably, a human, serves to alleviate the symptoms of or serves to cure a respiratory disease in the mammal. The preferred diseases to be treated include cystic fibrosis, asthma, emphysema, idiopathic pulmonary fibrosis and congenital surfactant deficiency. The preferred DNAs to be used therefore include the *CFTR* gene, an asthma gene, the  $\alpha$ 1AT gene, a gene affecting idiopathic pulmonary fibrosis and the SP-B and SB-C genes. The invention should also be construed to include biologically active fragments of each of these genes and should further be construed to include all forms of DNA, including cDNA, genomic DNA and synthetic DNA.

By the term "biologically active," as used herein, is meant a fragment of DNA which encodes a polypeptide that retains the biological activity of the full length protein from which the polypeptide is derived.

Using *CFTR* as an example, but understanding that the invention is not limited thereto, biologically active fragments of an isolated nucleic acid encoding *CFTR* will ordinarily be at least about 240 contiguous nucleic acids in length, typically at least about 500 contiguous nucleic acids, more typically at least about 1000 continuous nucleic acids, and even more typically, at least about 4000 to about 6000 contiguous nucleic acids in length. A fragment of nucleic acid encoding *CFTR* must be biologically active in order to be useful in the methods of the invention. The biological activity of *CFTR* is defined as an increase in efflux of chloride ions in epithelial cells following stimulation of the cells with either forskolin or cyclic AMP. In addition to the measurement of chloride ion efflux, *CFTR* activity may also be identified by measuring iodine efflux or efflux of other

encoding additional polypeptide sequence and optionally having promoter/regulatory sequences fused thereto to enhance or control expression of a protein encoded thereby.

5 The isolated nucleic acid should be construed to include a DNA or RNA sequence specifying the desired DNA or RNA, and any modified forms thereof, including modifications of the DNA or RNA which render the nucleotide sequence more stable when it is cell free or when it is associated with a cell. Modifications of nucleic acids may also be used to enhance the efficiency with which a nucleic sequence is taken up by a cell or the efficiency with which it is  
10 expressed in a cell. Any and all combinations of modifications of the nucleotide sequences are contemplated in the present invention.

The invention should not be construed as being limited solely to DNA or RNA encoding the aforementioned genes. Once armed with the present invention, it is readily apparent to one skilled in the art that DNA or RNA  
15 molecules which are homologous to the aforementioned genes and which encode proteins or peptides which are substantially similar in function to the function of the proteins encoded by the aforementioned genes may be obtained by following the well known procedures described in the art for the isolation of DNA or RNA molecules which are homologous to known DNA or RNA molecules.

20 "Homologous" as used herein, refers to the subunit sequence similarity between two polymeric molecules, *e.g.*, between two nucleic acid molecules, *e.g.*, two DNA molecules or two RNA molecules, or between two polypeptide molecules. When a subunit position in both of the two molecules is occupied by the same monomeric subunit, *e.g.*, if a position in each of two DNA  
25 molecules is occupied by adenine, then they are homologous at that position. The homology between two sequences is a direct function of the number of matching or homologous positions, *e.g.*, if half (*e.g.*, five positions in a polymer ten subunits in length) of the positions in two compound sequences are homologous then the two sequences are 50% homologous, if 90% of the positions, *e.g.*, 9 of 10, are matched  
30 or homologous, the two sequences share 90% homology. By way of example, the DNA sequences 3'ATTGCC5' and 3'TATGCG5' share 50% homology.

for expression of the product therefrom. In addition, the DNA molecule may include an origin of DNA replication which confers on the DNA the ability to replicate in the cell in which it has been introduced. Such origins of DNA replication are preferably eukaryotic replication origins and include any origin of DNA replication which facilitate replication of the DNA in a cell. Eukaryotic origins of DNA replication include, but are not limited to, the Epstein Barr virus DNA replication origin and associated DNA elements for facilitating replication, and the SV40 origin of replication. The ligation of an origin of DNA replication to a desired DNA molecule is well known in the art and is described, for example, in Sambrook et al. (1989, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor, NY). DNA which comprises an origin of DNA replication is useful in that it may have a longer therapeutic effect in an animal to which it is administered than DNA which does not comprise such an origin.

The DNA molecule to be transfected into cells, whether or not it is accompanied by additional sequences, is referred to herein as "an isolated nucleic acid molecule." It will be appreciated that as technology for the isolation of and modification of RNA advances, it may be possible to use an RNA molecule in place of the DNA molecule described herein. Thus, the term "isolated nucleic acid" should be construed to encompass both DNA and RNA.

By "isolated nucleic acid" as used herein is meant a nucleic acid sequence, a DNA or RNA sequence, which has been separated from the sequences which flank it in a naturally occurring state, *e.g.*, a DNA or RNA fragment which has been removed from the sequences which are normally adjacent to the fragment, *e.g.*, the sequences adjacent to the fragment in a genome in which it naturally occurs. The term also applies to nucleic acids which have been substantially purified from other components which naturally accompany the nucleic acid (*e.g.*, RNA, DNA or protein) in its natural state. The term therefore includes, for example, a recombinant DNA which is incorporated into a vector or into an autonomously replicating plasmid, or as a cDNA or a genomic or cDNA fragment produced by PCR or restriction enzyme digestion, independent of other sequences. It also includes a recombinant DNA which is part of a hybrid gene

histamine, and the like. Thus, any gene having the desired effect of alleviating asthma is included in the definition of an asthma gene as used herein.

When the disease to be treated is idiopathic pulmonary fibrosis, a DNA molecule, or a biologically active fragment thereof, comprising a gene whose protein product serves to alleviate idiopathic pulmonary fibrosis will be used.

By the term "a gene affecting idiopathic pulmonary fibrosis" as used herein, is meant a gene which is involved in the events leading to the onset or maintenance of this disease. Similar to the situation with respect to asthma, any gene having the desired effect of alleviating idiopathic pulmonary fibrosis is included in the definition of a gene affecting idiopathic pulmonary fibrosis as used herein.

In the case of emphysema, DNA comprising  $\alpha$ 1AT, or a biologically active fragment thereof, will be used to transfect cells and, in the case of congenital deficiency of surfactant protein, DNA comprising SP-B and/or SP-C will be used.

The various sources of known DNA's which may be used are either described or are referenced herein. For example, a source of CFTR DNA is described herein in the experimental examples section. A source of  $\alpha$ 1AT DNA is referenced in Canonico (*supra*) and a source of SP-B and SP-C DNAs is referenced in Whitsett et al. (1995, Physiological Reviews 75:749-757) and Nogee et al. (1994, J. Clin. Invest. 93:1860-1863). The source of other DNA's which may be used will be apparent to those of skill in the art of respiratory disease.

It will be appreciated that when the cells to be transfected are cells in culture, whether they are macrophages, tumor cells, fibroblast cell cultures or any other cell type, the DNA which will be used to transfect the cells will depend upon the particular application desired.

The DNA molecule may be contained within a plasmid, or may simply comprise the sequences to be transfected and any additional nucleic acid sequences which render the DNA more stable, or which enhance expression of the desired gene. Thus, the DNA molecule may include other sequences which enhance the expression of the DNA in a cell in which it has been introduced. For example, the DNA molecule may include promoter sequences, as described herein,

glycosyl residues results in high efficiency transfection of cells. Preferably, substitution of about 12% to about 40% of the amino groups of the polylysine molecule with glycosyl residues is optimal for transfection of cells.

It has been discovered in the present invention that the efficiency by which airway epithelial cells are transfected with nucleic acid complexed with substituted polylysine is affected by the weight to weight ratio of polylysine to DNA. Thus, in the case of polylysine substituted with lactose, preferably, the weight to weight ratio of lactosylated polylysine to DNA ranges from about one to one to about fifteen to one. More preferably, the weight to weight ratio of lactosylated polylysine to DNA ranges from about two to one to about nine to one. Even more preferably, the weight to weight ratio of lactosylated polylysine to DNA ranges from about three to one to about nine to one. When polylysine is substituted with sugars other than lactose, it will be appreciated that the weight to weight ratio of substituted polylysine to DNA will vary depending upon the type of sugar and the type and/or the size of the DNA being used. It is anticipated that the weight to weight ratio of substituted polylysine to DNA will be generally within the ranges given for lactosylated polylysine; however, the invention should not be construed as being limited to these ratios when glycosyl sugars other than lactose are used.

When transfection of cells *in vivo* in a mammal is contemplated, the type of DNA to be transfected will depend upon the disease to be treated. For example, for treatment of CF, a DNA molecule comprising the *CFTR* gene, or a biologically active fragment thereof, will be used. When the disease to be treated is asthma, a DNA molecule, or a biologically active fragment thereof, comprising a gene whose protein product serves to alleviate asthma will be used.

By the term "an asthma gene" as used herein, is meant a gene whose protein product has the effect of alleviating asthma. Such genes include those involved in the cascade of events leading to an asthma attack, as well as known or heretofore unknown genes which may be involved in the susceptibility of some individuals to asthma. Genes which are involved in the asthma cascade include genes encoding cytokines, such as, but not limited to, IL-4 and IL-5, genes affecting leukotriene synthesis, genes encoding proteins which govern the production of

of substituted polylysine derivatives is provided herein in the experimental details section.

To form a substituted polylysine DNA complex, the substituted polylysine derivative is added to DNA in a controlled manner in order that precipitation of the complex so formed does not occur. Generally, an amount of substituted polylysine is added to DNA in solution at a rate of several microliters per about 30 to about 60 seconds. Although, the examples provided in the experimental details section herein recite the rate of addition of substituted polylysine to DNA as being 15  $\mu$ l per 30 to 60 seconds, it will be appreciated that this rate may vary depending upon the precise amounts and types of substituted polylysine and DNA being mixed.

The types of substituted polylysine which are suitable for transfection of cells will vary depending on the cells to be used. When the polylysine DNA complex is to be used for transfection of airway epithelial cells *in vivo* in a mammal, preferably a human, then lactosylated polylysine is the polylysine derivative of choice. In contrast, in the case of immortalized airway epithelial cells, lactose,  $\alpha$ -glucose,  $\beta$ -galactose, mannose, mannose-6-phosphate, fucose or N-acetylglucosamine substituted polylysine may be used. Similarly, as will be described herein in greater detail, when cells which are not airway epithelial cells are to be transfected, glycosyl residues other than lactose may be used.

By the use of the term "glycosylated polylysine" as used herein, is meant a polylysine molecule which has substituted thereon glycosyl moieties. Thus, the term "glycosylated polylysine" may be distinguished from the term "gluconoylated polylysine" since a gluconoylated polylysine molecule has substituted thereon gluconoyl moieties. Glycosyl moieties differ from gluconoyl moieties in that glycosyl moieties comprise sugar, *i.e.*, carbohydrate molecules, whereas gluconoyl moieties are not considered in the art to be carbohydrate molecules since the characteristic ring structure is open. There are no gluconoyl moieties on the glycosylated polylysine molecules of the present invention.

It has been discovered in the present invention that substitution of about 10% to about 60% of the amino groups of the polylysine molecule with



were previously transfected with wild type CFTR and were subsequently transfected with pCMV*Luc*/lactosylated polylysine at a DNA to lactosylated polylysine ratio of 1:3 and the indicated concentrations of chloroquine with or without 5% glycerol.

5

#### DETAILED DESCRIPTION OF THE INVENTION

It has been discovered that substituted polylysines are capable of facilitating gene delivery to airway epithelial cells thereby providing an alternative to viral vectors for the delivery of genes into airway epithelial cells in patients having respiratory disease. Until the present invention, it was not known that airway epithelial cells could be transfected with DNA complexed to substituted polylysine. The substituted polylysines useful in the present invention comprise polylysine which is partially neutralized by blocking a number of positive-charged residues with sugar groups.

10

It has been discovered that polylysine substituted with  $\alpha$ -glucose,  $\beta$ -galactose or lactose are equally efficient as DNA transfer vehicles for the introduction of DNA into immortalized airway epithelial cells. However, polylysine substituted with lactose is superior to other substituted polylysines for effecting transfer of DNA into primary airway epithelial cells.

15

Thus, the invention includes a substituted polylysine which comprises either mono or disaccharide residues on a specified number of amino groups.

The use of polylysine substituted with mono or disaccharides for gene delivery is superior compared with other polylysine derivatives and compared with viral vectors in that mono or disaccharide substituted polylysine is non-immunogenic (Levine, 1964, *Proc. Soc. Exp. Biol. Med.* **116**:1127-1131; Fiume *et al.*, 1994, *Biochem. Pharmacol.* **47**:643-650).

The generation of substituted polylysine derivatives is well known in the art and is described, for example, in Midoux *et al.* (*supra*) and in Martinez-Fong *et al.*, 1994, *Hepatology*, **20**:1602-1608). A detailed description of the generation

20

(1994, *Biochim. Biophys. Acta* **1195**:96-102). Figure 15A (Panel A) comprises data obtained from T-84 tumor cells which are the prototype of CFTR-containing cells. Figure 15B (Panel B) comprises data obtained from CFT1 cells whose defect has been corrected by the addition of wild type CFTR. CF/T43 cells exhibited no response when similarly treated.

**Figure 16** is a series of images of photomicrographs demonstrating high efficiency expression of  $\beta$ -galactosidase following transfection with pCMVLacZ. The efficiency of gene transfer into CF/T43 cells was examined using 60  $\mu$ g of lactosylated polylysine complexed to 20  $\mu$ g of pCMVLacZ in the presence of 100  $\mu$ M chloroquine and 5% glycerol. The cells were transfected for four hours at 37°C either once or three times on three sequential days, followed by incubation in growth medium. Expression of LacZ was detected following fixation in 2% paraformaldehyde/0.2% glutaraldehyde followed by incubation for 18 hours at 37°C in X-gal stain. The cells were subsequently examined in a Nikon Diaphot 300 microscope (12.5 X magnification). Figure 16A is an image of cells transfected with pCMVLacZ coupled to lactosylated polylysine, wherein the cells were transfected for three consecutive days. Figure 16B is an image of cells transfected as in Figure 16A, wherein the cells were transfected once. Figure 16C is an image of cells transfected as in Figure 16A, wherein the cells were transfected with the plasmid but without lactosylated polylysine.

**Figure 17** is a series of images of photomicrographs of cells depicting expression of CFTR in CF airway epithelial cells in primary culture. Expression of CFTR was detected by *in situ* hybridization. Figure 17A depicts primary cells grown on coverslips which were transfected with pAdCFTR complexed with lactosylated polylysine. Figure 17B depicts primary cells grown on coverslips which were transfected with pAdCFTR which was not complexed to lactosylated polylysine.

**Figure 18** is a series of graphs depicting the effect of potentiating agents on human airway epithelial cell lines. Figure 18A: BEAS2B, immortalized nonCF airway epithelial cells.; Figure 18B: CF/T1 (wild type) immortalized airway epithelial cells obtained from a CF patient having the  $\Delta$ F508 mutation. These cells

**Figure 10** is a graph depicting enhancement of reporter gene expression in CF/T43 cells transfected with a plasmid encoding the reporter gene, pCMVLuc, complexed to lactosylated polylysine, wherein the transfection was conducted in the presence (open circles) or absence (closed circles) of 100  $\mu$ M chloroquine and the indicated concentrations of glycerol.

**Figure 11** is a graph depicting enhancement of reporter gene expression in primary airway epithelial cells transfected with a plasmid encoding the reporter gene, pCMVLuc, complexed to lactosylated polylysine, wherein the transfection was conducted in the presence of the indicated concentrations of glycerol without the addition of chloroquine. A transfection assay conducted in the presence of 50  $\mu$ M chloroquine was used for comparison.

**Figure 12** is a graph depicting enhancement of reporter gene expression in CF/T43 cells transfected with a plasmid encoding the reporter gene, pCMVLuc, complexed to gluconoylated polylysine, in the presence of the indicated concentrations of fusogenic peptide or chloroquine.

**Figure 13** is a graph depicting the enhancement of reporter gene expression in CF/T43 cells transfected with a plasmid encoding the reporter gene pCMVLuc coupled to lactosylated polylysine in the presence of the indicated concentrations of different additives as noted.

**Figure 14** is a graph depicting the enhancement of reporter gene expression in CF/T43 cells transfected with pCMVLuc coupled to different concentrations of several preparations of lactosylated polylysines. PM 186 is presented as the mean value  $1.08 \pm 0.02$  RLU ( $\times 10^8$ ;  $n = 10$ ).

**Figure 15** is a series of graphs depicting assays for  $\text{Cl}^-$  efflux using  $^{125}\text{I}$ . A concentration of  $2 \times 10^5$  cells were cultured in 15 mm wells for three to four days. The cells were washed and  $^{125}\text{I}$  was added in efflux medium, following which, 1 ml fractions were obtained from the cultures. A mixture comprising 20 mM forskolin, 250 mM cAMP and 500 mM isobutyl-1-methylxanthine. (IBMX) was added to the cultures at the time indicated by the arrow, and removal of 1 ml fractions from the cultures at the indicated times was continued. Efflux was measured and was expressed as the rate per minute as described in Santos et al.

polylysines complexed with pCMV*Luc* and the level of gene expression in these cells was compared with that in cells transfected with gluconoylated polylysine complexed with pCMV*Luc*. Following transfection in the presence of 100  $\mu$ M chloroquine, the cells were incubated in LHC-9 medium (Biofluids Inc.) and the amount of luciferase activity expressed in the cells was measured. The results in **Figure 6A** and **Figure 6B** are those obtained from two separately conducted experiments in two different primary cultures of cells.

**Figure 7**, comprising parts A and B, is a series of graphs depicting reporter gene expression in non-CF tracheal cells in primary culture using glycosylated polylysines as the vehicle. Cells were transfected with glycosylated polylysines complexed with pCMV*Luc* and the level of gene expression in these cells was compared with that in cells transfected with gluconoylated polylysine complexed with pCMV*Luc*. Following transfection in the presence of 100  $\mu$ M chloroquine, the cells were incubated in LHC-9 medium and the amount of luciferase activity in the cells was measured. The results in **Figure 7A** and **Figure 7B** are those obtained from two separately conducted experiments in two different primary cultures of cells.

**Figure 8** is a graph depicting the effect of chloroquine on reporter gene expression in non-CF tracheal cells in primary culture. Cells were transfected for 4 hours with lactosylated polylysine and pCMV*Luc* at a ratio of 3:1 and the indicated concentrations of chloroquine. Following transfection, cells were incubated for 48 hours and the amount of luciferase activity in the cells was subsequently measured.

**Figure 9** is a series of photomicrographs depicting binding of Lac-BSA-FITC to CF/T43 cells. CF/T43 cells were grown on coverslips for 24 hours, the culture medium was removed and the cells were processed for the binding assay. Lac-BSA-FITC (100  $\mu$ g per ml) was added to the cells for 30 minutes at 4°C in the absence of (**Figure 9A**), and the presence of (**Figure 9B**) 0.1 M lactose. The cells were examined in a Nikon Diaphot 300 microscope (Magnification is X 250).

The amount of luciferase expressed in the cells was then measured. When cells were transfected for 2 hours,  $8.5 \times 10^6$  RLU per mg of protein was detected.

In **Figure 3B**, CF/T43 cells were transfected for 4 hours with the plasmid pCMV*Luc* and gluconoylated polylysine. Following transfection, the cells were washed and were incubated in KGM medium for the times indicated on the figure. At those indicated times, the amount of luciferase expressed in the cells was assessed. At 6 and 12 hours following the 4 hour transfection period,  $3 \times 10^4$  and  $1.3 \times 10^5$  RLU, respectively, were detected per well.

**Figure 4** is a graph depicting reporter gene expression in cells transfected with various glycosylated polylysines as vehicles. The substituted polylysines indicated on the figure were complexed with 5  $\mu$ g of the reporter plasmid pCMV*Luc*. CF/T43 cells were transfected for 4 hours, washed and incubated in KGM medium and luciferase activity was measured. "None" indicates that non substituted polylysine was added to the transfection mixture; "GlcA" is gluconoylated polylysine.

**Figure 5**, comprising parts A, B and C, is a series of graphs depicting optimization of expression of a reporter gene in transfected CF/T43 cells using lactosylated polylysine as a vehicle.

In **Figure 5A**, one  $\mu$ g of pCMV*Luc* was used in the presence of 100  $\mu$ M chloroquine while the concentration of lactosylated polylysine was varied as indicated.

In **Figure 5B**, the time of the actual transfection was varied from 2 to 10 hours using 3  $\mu$ g lactosylated polylysine and 1  $\mu$ g of reporter gene pUT 650 (*i.e.*, in a w/w ratio of 3:1) in the presence of 100  $\mu$ M chloroquine.

**Figure 5C**. Chloroquine at the indicated concentrations was added to the transfection medium containing lactosylated polylysine/pUT 650 complex at a ratio of 3:1 for 4 hours. Cells were washed, incubated in KGM medium for 48 hours, and luciferase activity was measured. In the absence of chloroquine,  $2.5 \times 10^6$  RLU per mg of protein was expressed in the cells.

**Figure 6**, comprising parts A and B, is a series of graphs depicting reporter gene expression in CF airway epithelial cells in primary culture using glycosylated polylysines as the vehicle. Cells were transfected with glycosylated

## BRIEF DESCRIPTION OF THE DRAWINGS

**Figure 1** is a graph depicting the influence of chloroquine on gene expression. Chloroquine at the indicated concentrations, was added to transfection medium containing gluconoylated polylysine and the plasmid pUT 650. The mixture was added to CF/T43 cells which were then incubated for 4 hours. Following subsequent culture of the cells for 48 hours in KGM medium (Clonetics Corp.), the cells were lysed and the level of expression of luciferase was assessed using a luminometer. The relative light units (RLU) were measured at 5 s and the amount of RLU per mg of protein was calculated. When cells were incubated in the absence of chloroquine,  $6.8 \times 10^5$  RLU per mg of protein was detected.

**Figure 2**, comprising parts A and B, is a series of graphs depicting the relationship between the concentration of plasmid and cell number and transfected gene expression.

In **Figure 2A**, a transfection assay was performed as described in Figure 1 wherein the amount of gluconoylated polylysine:pUT 650 (2:1, weight/weight) was varied as indicated, in relationship to the cell number which was maintained at a constant value of  $1.5 \times 10^5$  cells per culture well. When no plasmid was present in the mixture, a blank value was obtained.

In **Figure 2B**, a transfection assay was performed as described in Figure 1, wherein a constant amount (1  $\mu$ g) of the plasmid, pCMV*Luc* and gluconoylated polylysine (2  $\mu$ g) was added to a varying number of CF/T43 cells per well as indicated in the figure.

**Figure 3**, comprising parts A and B, is a series of graphs depicting the effect of transfection time on luciferase gene expression (A) and the effect of incubation time post transfection on luciferase gene expression (B) following transfection of CF/T43 cells using gluconoylated polylysine as a vehicle.

In **Figure 3A**, the level of expression of the reporter gene, pUT 650, was assessed at various times post-transfection of CF/T43 cells. Chloroquine (100  $\mu$ M) was included in the transfection mixture. The DNA complex was added to CF/T43 cells in the transfection medium and at various times the transfection mixture was removed and the cells were incubated in KGM medium for 48 hours.

The kit may further comprise a reporter DNA and may also further comprise at least one of chloroquine, glycerol and a fusogenic peptide.

Preferably, the glycosylated polylysine in the kit has a sugar component selected from the group consisting of lactose,  $\alpha$ -glucose,  $\beta$ -galactose, mannose, mannose-6-phosphate, fucose and N-acetylglucosamine.

Also preferably, the reporter DNA is selected from the group consisting of a chloramphenicol acetyl transferase gene, a luciferase gene, a green fluorescent protein gene, and a  $\beta$ -galactosidase gene.

The invention also includes a nebulizer having a composition comprising a complex comprising an isolated nucleic acid and a glycosylated polylysine placed therein. Preferably, the isolated nucleic acid is DNA encoding CFTR and the glycosylated polylysine is lactosylated polylysine.

In addition, the invention includes a bronchoscope having a composition comprising a complex comprising an isolated nucleic acid and a glycosylated polylysine placed therein. Preferably, the isolated nucleic acid is DNA encoding CFTR and the glycosylated polylysine is lactosylated polylysine.

There is further included in the invention an airway epithelial cell transfected with a complex comprising an isolated nucleic acid and a glycosylated polylysine. Preferably, the isolated nucleic acid is DNA encoding CFTR and the glycosylated polylysine is lactosylated polylysine.

In addition, the invention includes a composition for transfection of airway epithelial cells comprising a complex comprising an isolated nucleic acid and a lactosylated polylysine, wherein the isolated nucleic acid is DNA selected from the group consisting of DNA encoding CFTR, an asthma gene, DNA encoding  $\alpha$ 1AT, a gene affecting idiopathic pulmonary fibrosis, DNA encoding SP-B and DNA encoding SP-C. The composition may further at least one of chloroquine, glycerol and a fusogenic peptide.

In a preferred embodiment, the DNA is DNA encoding CFTR.

Preferably, the airway epithelial cells are transfected *in vitro* or are transfected *in vivo*.

acid encoding CFTR, or a biologically active fragment thereof, and a glycosylated polylysine. The composition may further comprise at least one of chloroquine, glycerol and a fusogenic peptide.

5 In a preferred embodiment of this aspect of the invention, the pharmaceutical composition is administered to the human by a means selected from the group consisting of aerosol nebulizer, bronchoscopy and injection *in utero*.

In yet another preferred embodiment of this aspect of the invention, the isolated nucleic acid comprises DNA, preferably, cDNA.

10 In another aspect of this method of the invention, the glycosylated polylysine has a sugar component selected from the group consisting of lactose,  $\alpha$ -glucose,  $\beta$ -galactose, mannose, mannose-6-phosphate, fucose and N-acetylglucosamine. Preferably, the glycosylated polylysine comprises lactosylated polylysine. More preferably, about 10% to about 60% of the amino groups of the polylysine have a lactose molecule substituted thereon. Also more preferably, the  
15 weight to weight ratio of lactosylated polylysine to DNA in the complex is about one to one to about fifteen to one. Even more preferably, the weight to weight ratio of lactosylated polylysine to DNA in the complex is about three to one to about nine to one.

20 There is further included in the invention a method of identifying a test compound capable of modulating the activity of CFTR. The method comprises transfecting airway epithelial cells in the presence or absence of the test compound with a complex comprising an isolated nucleic acid encoding CFTR, or a biologically active fragment thereof, and a glycosylated polylysine, and measuring the activity of CFTR in the cells, wherein a higher or a lower level of CFTR activity  
25 in the presence of the test compound compared with CFTR activity in cells in the absence of the test compound is an indication that the test compound is capable of modulating the activity of CFTR.

The invention includes a compound identified according to the just described method.

30 In addition, the invention relates to an *in vitro* cell transfection kit comprising a selection of glycosylated polylysines and instructions for using the kit.



In addition, the glycosylated polylysine in the pharmaceutical composition may have a sugar component selected from the group consisting of lactose,  $\alpha$ -glucose,  $\beta$ -galactose, mannose, mannose-6-phosphate, fucose and N-acetylglucosamine. Preferably, the glycosylated polylysine comprises lactosylated polylysine. More preferably, about 10% to about 60% of the amino groups of the polylysine have a lactose molecule substituted thereon. Even more preferably, about 12% to about 40% of the amino groups of the polylysine have a lactose molecule substituted thereon.

In another preferred embodiment, the weight to weight ratio of lactosylated polylysine to DNA in the complex is about one to one to about fifteen to one.

The invention also includes a lactosylated polylysine nucleic acid complex comprising DNA encoding CFTR, or a biologically active fragment thereof, and lactosylated polylysine, wherein about 10% to about 60% of the amino groups of the polylysine have a lactose molecule substituted thereon, and the weight to weight ratio of lactosylated polylysine to the DNA in the complex is about one to one to about fifteen to one, the complex being capable of transfecting airway epithelial cells when added thereto. Preferably, the weight to weight ratio of lactosylated polylysine to the isolated nucleic acid in the complex is about nine to one.

Also included in the invention is a kit comprising an isolated nucleic acid encoding CFTR, or a biologically active fragment thereof, a glycosylated polylysine and instructions for using the kit for transfection of airway epithelial cells.

In addition, the invention includes a kit comprising an isolated nucleic acid encoding CFTR, or a biologically active fragment thereof, a glycosylated polylysine and instructions for using the kit for treatment of cystic fibrosis in a human patient.

The invention further relates to a method of treating a human patient having cystic fibrosis, the method comprising administering to the human a pharmaceutical composition comprising a complex comprising an isolated nucleic

The pharmaceutical composition may further comprise at least one of chloroquine, glycerol and a fusogenic peptide.

In one embodiment, the respiratory disease is selected from the group consisting of cystic fibrosis, asthma, emphysema, idiopathic pulmonary  
5 fibrosis and congenital deficiency of surfactant protein.

In another embodiment, DNA comprises cDNA, which preferably encodes CFTR.

In yet another embodiment, the glycosylated polylysine has a sugar component selected from the group consisting of lactose,  $\alpha$ -glucose,  $\beta$ -galactose,  
10 mannose, mannose-6-phosphate, fucose and N-acetylglucosamine. Preferably, the glycosylated polylysine comprises lactosylated polylysine.

In a preferred embodiment, about 10% to about 60% of the amino groups of the polylysine have a lactose molecule substituted thereon. More preferably, about 12% to about 40% of the amino groups of the polylysine have a  
15 lactose molecule substituted thereon.

In yet another preferred embodiment, the weight to weight ratio of lactosylated polylysine to DNA in the complex is about one to one to about fifteen to one.

There is further provided in the invention a pharmaceutical  
20 composition for treatment of a respiratory disease in a human comprising a complex comprising an isolated nucleic acid and a glycosylated polylysine, wherein the isolated nucleic acid comprises antisense DNA capable of inhibiting the expression of a gene, which gene is required for the development of a respiratory disease in a mammal.

25 In one embodiment of this aspect of the invention, gene is selected from the group consisting of an interleukin gene and a gene affecting leukotriene synthesis. Preferably, the respiratory disease is asthma and the gene is a gene encoding IL-4 or IL-5.

The pharmaceutical composition may further comprise at least one  
30 of chloroquine, glycerol and a fusogenic peptide.

a complex comprising an isolated nucleic acid and a glycosylated polylysine, and adding the complex to the airway epithelial cells.

In one aspect, the composition further comprises at least one of chloroquine, glycerol and a fusogenic peptide.

5 In another aspect, the glycosylated polylysine has a sugar component selected from the group consisting of lactose,  $\alpha$ -glucose,  $\beta$ -galactose, mannose, mannose-6-phosphate, fucose and N-acetylglucosamine. Preferably, the glycosylated polylysine is lactosylated polylysine.

10 In one embodiment, the cells are transfected *in vitro* and in another embodiment, the cells are transfected *in vivo*.

In yet other embodiments, the isolated nucleic acid is DNA or cDNA. The DNA may be selected from the group consisting of DNA encoding CFTR, an asthma gene, DNA encoding  $\alpha$ 1AT, a gene affecting idiopathic pulmonary fibrosis, DNA encoding SP-B and DNA encoding SP-C. Preferably,  
15 the DNA encodes CFTR.

In another embodiment, the DNA is antisense DNA capable of inhibiting the expression of a gene, which gene is required for the development of a respiratory disease in a mammal.

20 In preferred embodiments, the gene is selected from the group consisting of an interleukin gene and a gene affecting leukotriene synthesis. More preferably, the respiratory disease is asthma and the gene is a gene encoding IL-4 or a gene encoding IL-5.

The invention also includes a pharmaceutical composition for treatment of a respiratory disease in a human. The composition comprises a  
25 complex comprising an isolated nucleic acid encoding a protein, or a biologically active fragment thereof, and a glycosylated polylysine, wherein the isolated nucleic acid is DNA selected from the group consisting of DNA encoding CFTR, an asthma gene, DNA encoding  $\alpha$ 1AT, a gene affecting idiopathic pulmonary fibrosis, DNA encoding SP-B and DNA encoding SP-C, the complex being suspended in a  
30 pharmaceutically acceptable carrier, the complex being capable of transfecting airway epithelial cells when added thereto.

Physiological Reviews 75:749-757) and Noguee et al. (1994, J. Clin. Invest. 93:1860-1863).

As an alternative to virus or lipid mediated gene therapy, it has been reported that substitution of polylysine with lactose residues facilitates a high level of transfection of HepG2 cells via galactose-specific membrane lectins (Midoux *et al.*, 1993, *Nucleic Acids Res.* **21**:871-878; Erbacher *et al.*, 1995, *Bioconj. Chem.* **6**:401-410). It is also known that partially gluconoylated polylysine is an efficient vehicle for reporter gene expression in a number of different cell types (Midoux *et al.*, 1995, *International Application Publication No. WO 95/30020*; U.S. Patent No. 5,595,897).

Polylysine substituted with specific sugars such as mannose or fucose may be used to transfect human macrophages which have a membrane lectin for mannose and fucose (Erbacher *et al.*, 1996, *Hum. Gene Ther.* **7**:721-729). Further, complex asialo-oligosaccharides coupled to short polylysine polymers have been used to transfect DNA into HepG2 cells (Wadhwa *et al.*, 1995, *Bioconj. Chem.* **6**:283-291).

There remains an acute need for a suitable vehicle for delivery of genes to respiratory cells, which vehicle must be non-immunogenic. Given the paucity of information on the nature of endogenous lectins on human airway epithelial cells (Drickamer *et al.*, 1993, *Ann. Rev. Cell Biol.* **9**:237-264), the use of polylysine derivatized with specific carbohydrates for delivery of genes to airway epithelial cells could not be predicted to successfully facilitate introduction of genes into these cells.

## SUMMARY OF THE INVENTION

The invention relates to a method of transfecting airway epithelial cells comprising adding to the cells a composition comprising a complex comprising an isolated nucleic acid and a glycosylated polylysine.

There is also provided in the invention a method of transfecting airway epithelial cells, the method comprising generating a composition comprising

physiological correction of the deficiency. Adenovirus-mediated gene therapy directed to lung cells has been attempted. However, because of the problems associated with adenovirus-induced inflammation, this is not the preferred approach. The use of other viruses and of liposomes has also been contemplated as  
5 a means of delivering  $\alpha$ 1AT to lung cells (Canonico, *supra*).

Idiopathic pulmonary fibrosis (IPF) is a lethal disease with a median time from diagnosis to death of 3 to 5 years. Since, current therapies for IPF have marginal effect on improved lung function or overall survival, a gene therapy approach for treatment of this disease is justified. In IPF, an inflammatory response  
10 to an unidentified insult or injury occurs following an exuberant fibrotic response. The initial inflammatory response is predominantly neutrophilic but evolves to a predominant lymphocytic and monocytic response. As yet, no specific genetic defect has been identified; however, gene therapy targeted to specific sites in the disease pathway, has been contemplated. For example, antisense therapy targeting  
15 specific growth factors or cytokines implicated in IPF has been proposed, in addition to delivery of other genes such as the cyclo-oxygenase-2 gene, the latter of which may block the effects of certain proinflammatory cytokines (Canonico, *supra*).

Congenital deficiency of surfactant protein results in severe  
20 respiratory disease in infants. The fundamental importance of surfactant protein (SP)-B in pulmonary function has been elucidated from studies on infants unable to produce SP-B due a genetic defect which gives rise to a lethal neonatal respiratory disease. Respiratory failure in these infants was refractory to therapies which included mechanical ventilation, surfactant replacement and extracorporeal  
25 membrane oxygenation. A genetically based deficiency in production of a second surfactant protein, SP-C, may also contribute to the development of this disease. Since this disease is governed by genes which have been identified and in view of the absence of any effective current therapy for this disease, a gene therapy approach for treatment of SP-B and/or SP-C deficiency seems appropriate. For a  
30 discussion on congenital deficiency of surfactant protein, see Whitsett et al. (1995,

which ultimately leads to bronchioconstriction. IL-4 induces production by activated B lymphocytes of immunoglobulin (Ig) E which, in turn, induces the production of histamine from mast cells. IL-5 triggers the production by eosinophils of small fatty molecules known as leukotrienes. The combined action of histamine and leukotrienes causes blood vessels to leak and lung tissues to swell. The smooth muscles of the airways constrict and mucus production is induced which serves to further clog the already constricted airways.

Current asthma therapy is aimed at treating the end result, *i.e.*, the airway constriction. However, targets other than the end point may be more amenable to therapy, particularly gene therapy. In addition, asthma is believed to have a genetic component, and in fact, the identification of an asthma gene has recently been announced (Vogel, 1997, Science 276:1327). This disease is therefore suitable for treatment using a gene therapy approach.

Alpha<sub>1</sub> antitrypsin ( $\alpha$ 1AT) deficiency, like CF, is an inherited monogenic disorder having virtually no effective therapy beyond treatment for alleviation of the symptoms of the disease.  $\alpha$ 1AT deficiency is primarily associated with emphysema, a lung disease characterized by unopposed elastolytic destruction of the lung parenchyma. Although  $\alpha$ 1AT is synthesized primarily in liver cells, functional  $\alpha$ 1AT is responsible for over 95% of the antiprotease protection in the lower respiratory system. The most common genetic abnormality associated with premature emphysema is the Z allele. In this mutant allele, a lysine is substituted for glutamic acid at amino acid position 342 in  $\alpha$ 1AT, thereby altering the three dimensional configuration of the protein and affecting secretion of the protein from the cells in which it is synthesized. Other mutant alleles of the  $\alpha$ 1AT gene also contribute to the disease, and irrespective of the genetic abnormality, a critical threshold of an  $\alpha$ 1AT serum level of less than 10  $\mu$ M appears necessary for an individual to develop pulmonary emphysema.

Both the liver and the lung have been targeted for gene therapy as a means of treating  $\alpha$ 1AT deficiency. With respect to the liver, although successful liver-directed  $\alpha$ 1AT gene therapy has been achieved using various strategies, serum  $\alpha$ 1AT levels in all of these systems were below what would be necessary for



## INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PC1/US 95/17005

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO-A-9533061	07-12-95	DE-A- 4418965 AU-B- 2615895	07-12-95 21-12-95
WO-A-9423050	13-10-94	AU-B- 6497894 EP-A- 0693130	24-10-94 24-01-96
WO-A-9220316	26-11-92	AU-B- 668870 AU-B- 2155092 CA-A- 2103064 EP-A- 0584279 JP-T- 6510524	23-05-96 30-12-92 15-11-92 02-03-94 24-11-94
WO-A-9304701	18-03-93	AU-B- 2678092 CA-A- 2116107 EP-A- 0666923 JP-T- 7500820	05-04-93 18-03-93 16-08-95 26-01-95
WO-A-9219749	12-11-92	AU-B- 1922092	21-12-92
US-A-5166320	24-11-92	JP-A- 63269985	08-11-88
WO-A-9205250	02-04-92	AU-B- 8628291 CA-A- 2092319 EP-A- 0556197 JP-T- 6503714	15-04-92 26-03-92 25-08-93 28-04-94
EP-A-0388758	26-09-90	AU-B- 637085 AU-B- 5137290 CA-A- 2012311 IL-A- 93755 JP-A- 3200800 US-A- 5354844	20-05-93 20-09-90 16-09-90 31-12-95 02-09-91 11-10-94
US-A-3725545	03-04-73	NONE	
FR-A-2122325	01-09-72	NONE	



FURTHER INFORMATION CONTINUED FROM PCT/ISA/210

- claims 1- 7 and 15-20: Nucleic acids condensed with a nucleic acid condensing agent comprising a polycation chemically conjugated with polyalkylene glycol and gene delivery vehicles comprising the same
- claims 8-14 and 21-26: Nucleic acids condensed with a nucleic acid condensing agent comprising a polycation chemically conjugated with a polysaccharide and gene delivery vehicles comprising the same
- claims 27-33 : Nucleic acids condensed with a polycation according to claim 27

## INTERNATIONAL SEARCH REPORT

International application No.

PCT/US95/17005

**Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)**

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.: 34-41  
because they relate to subject matter not required to be searched by this Authority, namely:  
Remark: Although these claims are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.
2. ☐ Claims Nos.:  
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

**Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)**

This International Searching Authority found multiple inventions in this international application, as follows:

- claims 1- 7 and 15-20
- claims 8-14 and 21-26
- claims 27-33

1. ☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☒ No protest accompanied the payment of additional search fees.

## INTERNATIONAL SEARCH REPORT

International Application No

PC1/US 95/17005

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US,A,3 725 545 (MAES, ROLAND) 3 April 1973 see the whole document ---	8-12, 21-24
X	J. VIROL. (1969), 4(4), 380-7 CODEN: JOVIAM, 1969, XP000569882 WARDEN, DAVID ET AL: "Influence of diethylaminoethyl- dextran on uptake and degradation of polyoma virus deoxyribonucleic acid by mouse embryo cells" see the whole document ---	8-12, 21-24
X	ARCH. BIOCHEM. BIOPHYS. (1971), 147(1), 85-98 CODEN: ABBIA4, 1971, XP000569881 HAVLIZA, D. ET AL: "Complex formation between poliovirus RNA and polycations" see the whole document ---	8-11,21, 22
X	MOL. REPROD. DEV., vol. 31, no. 3, 1992, pages 161-169, XP000569627 M. LAVITRANO ET AL.: "The interaction between exogenous DNA and sperm cells" see the whole document ---	8-12, 21-24
X	J. VIROL. (1971), 8(1), 35-40 CODEN: JOVIAM, 1971, XP000569880 WENTZKY, P. ET AL: "Influence of polycations on the interaction between poliovirus multistranded ribonucleic acid and HeLa cells" see the whole document ---	8-12, 21-24
X	PROC. SOC. EXP. BIOL. MED. (1966), 123(3), 939-45 CODEN: PSEBAA, December 1966, XP000569883 BACHRACH, HOWARD L.: "Ribonucleic acid of foot-and-mouth disease virus;an ultrasensitive plaque assay" see the whole document ---	8-10, 21-23
X	J. GEN. VIROL. (1971), 11(PT. 2), 111-17 CODEN: JGVIAV, 1971, XP000569879 HULL, R.: "Effect of DEAE- dextran on the nucleic acids of two plant viruses" see the whole document ---	8-10, 21-23
3 6	X FR,A,2 122 325 (R.F.E. MAES) 1 September 1972 see the whole document -----	8-10, 21-23

## INTERNATIONAL SEARCH REPORT

International Application No

PC/US 95/17005

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	PROC. NATL. ACAD. SCI. USA , vol. 87, May 1990, pages 3410-3414, XP002002759 E. WAGNER ET AL.: "Transferrin-polycation conjugates as carriers for DNA uptake into cells" see the whole document ---	1-7, 13-20, 25,26
Y	PROC. NATL. ACAD. SCI. USA, vol. 87, May 1990, pages 3655-3659, XP002002760 M. ZENKE ET AL.: "Receptor-mediated endocytosis of transferrin-polycation conjugates: An efficient way to introduce DNA into hematopoietic cells " see the whole document ---	1-7, 13-20, 25,26
Y	BIOCONJUGATE CHEM. , vol. 2, no. 4, 1991, pages 226-231, XP002002761 E. WAGNER ET AL.: "DNA-binding transferrin conjugates as functional gene-delivery agents: synthesis by linkage of polylysine or ethidium homodimer to the transferrin carbohydrate moiety" see the whole document ---	1-7, 13-20, 25,26
Y	BIOCHEMICA ET BIOPHYSICA ACTA, vol. 815, 1985, pages 515-518, XP002002762 K. ARNOLD ET AL.: "The dielectric properties of aqueous solutions of pol{ethylene glycol} and their influence on membrane structure" see the whole document ---	1-7, 15-20
Y	SYNTHETIC COMMUNICATIONS , vol. 22, no. 16, 1992, pages 2417-2424, XP002002763 T.P KOGAN: "The synthesis of substituted methoxy-poly{ethyleneglycol} derivatives suitable for selective protein modification" cited in the application see page 2417, paragraph 1 ---	1-7, 15-20
X	ARCH VIROL 55 (4). 1977 (RECD 1978) 275-286. CODEN: ARVIDF ISSN: 0304-8608, XP002002764 MAES R F ET AL: "POTENTIATION OF FOOT-AND-MOUTH DISEASE VACCINES WITH POLY CATIONIC NUCLEIC - ACID COMPLEXES." see the whole document ---	8-12, 21-24
	---	

-/--

## INTERNATIONAL SEARCH REPORT

International Application No

PC/US 95/17005

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO,A,93 04701 (UNIV CONNECTICUT) 18 March 1993	1-7, 13-20, 25,26
X	see the whole document	27,28, 31-33
Y	WO,A,92 19749 (REGENTS ACTING FOR AND ON BEHA ;UNIV CONNECTICUT (US); EINSTEIN CO) 12 November 1992	1-7, 13-20, 25,26
X	see the whole document	27,28, 31-33
Y	US,A,5 166 320 (WU GEORGE Y ET AL) 24 November 1992	1-7, 13-20, 25,26
X	see the whole document	27,28, 31-33
Y	WO,A,92 05250 (UNIV CONNECTICUT) 2 April 1992	1-7, 13-20, 25,26
X	see the whole document	27,28, 31-33
Y	PROC. NATL. ACAD. SCI. USA, vol. 88, October 1991, pages 8850-8854, XP000569906 D.T. CURIEL ET AL.: "Adenovirus enhancement of transferrin-polylysine-mediated gene delivery" cited in the application see the whole document	1-7, 13-20, 25,26
Y	EP,A,0 388 758 (BOEHRINGER INGELHEIM INT) 26 September 1990	1-7, 13-20, 25,26
X	see the whole document	27,28, 31-33
Y	PROC. NATL. ACAD. SCI. USA, vol. 88, May 1991, pages 4255-4259, XP002002758 E. WAGNER ET AL.: "Transferrin-polycation-DNA complexes: The effect of polycations on the structure of the complex and DNA delivery to cells" see the whole document	1-5, 13-20, 25,26

-/--

## INTERNATIONAL SEARCH REPORT

International Application No

PC, /US 95/17005

## A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 C12N15/87 A61K47/48 //C07H21/00

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C12N A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	WO,A,95 33061 (BOEHRINGER INGELHEIM INT ;BUSCHLE MICHAEL (AT); WAGNER ERNST (AT);) 7 December 1995 see the whole document ---	1-7, 15-20, 27,31-33
Y	WO,A,94 23050 (UNIV CONNECTICUT ;TARGETECH INC (US)) 13 October 1994	1-7, 13-20, 25,26
X	see the whole document ---	27,28, 31-33
Y	WO,A,92 20316 (UNIV CONNECTICUT ;TARGETECH INC (US)) 26 November 1992	1-7, 13-20, 25,26
X	see the whole document ---	27,28, 31-33
	---	

-/--



Further documents are listed in the continuation of box C.



Patent family members are listed in annex.

## \* Special categories of cited documents:

- \*A\* document defining the general state of the art which is not considered to be of particular relevance
- \*E\* earlier document but published on or after the international filing date
- \*L\* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- \*O\* document referring to an oral disclosure, use, exhibition or other means
- \*P\* document published prior to the international filing date but later than the priority date claimed

\*T\* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

\*X\* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

\*Y\* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

\*&\* document member of the same patent family

Date of the actual completion of the international search

10 July 1996

Date of mailing of the international search report

16. 07. 96

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2  
NL - 2280 HV Rijswijk  
Tel. (+ 31-70) 340-2040, Tx. 31 651 epo nl,  
Fax: (+ 31-70) 340-3016

Authorized officer

Hix, R

**FOR THE PURPOSES OF INFORMATION ONLY**

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AM	Armenia	GB	United Kingdom	MW	Malawi
AT	Austria	GE	Georgia	MX	Mexico
AU	Australia	GN	Guinea	NE	Niger
BB	Barbados	GR	Greece	NL	Netherlands
BE	Belgium	HU	Hungary	NO	Norway
BF	Burkina Faso	IE	Ireland	NZ	New Zealand
BG	Bulgaria	IT	Italy	PL	Poland
BJ	Benin	JP	Japan	PT	Portugal
BR	Brazil	KE	Kenya	RO	Romania
BY	Belarus	KG	Kyrgyzstan	RU	Russian Federation
CA	Canada	KP	Democratic People's Republic of Korea	SD	Sudan
CF	Central African Republic	KR	Republic of Korea	SE	Sweden
CG	Congo	KZ	Kazakhstan	SG	Singapore
CH	Switzerland	LI	Liechtenstein	SI	Slovenia
CI	Côte d'Ivoire	LK	Sri Lanka	SK	Slovakia
CM	Cameroon	LR	Liberia	SN	Senegal
CN	China	LT	Lithuania	SZ	Swaziland
CS	Czechoslovakia	LU	Luxembourg	TD	Chad
CZ	Czech Republic	LV	Latvia	TG	Togo
DE	Germany	MC	Monaco	TJ	Tajikistan
DK	Denmark	MD	Republic of Moldova	TT	Trinidad and Tobago
EE	Estonia	MG	Madagascar	UA	Ukraine
ES	Spain	ML	Mali	UG	Uganda
FI	Finland	MN	Mongolia	US	United States of America
FR	France	MR	Mauritania	UZ	Uzbekistan
GA	Gabon			VN	Viet Nam



## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

<b>(51) International Patent Classification <sup>6</sup> :</b> <b>C12N 15/87, A61K 47/48 // C07H 21/00</b>		<b>A3</b>	<b>(11) International Publication Number:</b> <b>WO 96/21036</b>
			<b>(43) International Publication Date:</b> 11 July 1996 (11.07.96)
<b>(21) International Application Number:</b> PCT/US95/17005			<b>(81) Designated States:</b> AM, AT, AU, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IS, JP, KE, KG, KP, KR, KZ, LK, LR, LT, LU, LV, MD, MG, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TT, UA, UG, UZ, VN, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).  <b>Published</b> <i>With international search report.</i> <i>Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>  <b>(88) Date of publication of the international search report:</b> 6 September 1996 (06.09.96)
<b>(22) International Filing Date:</b> 26 December 1995 (26.12.95)			
<b>(30) Priority Data:</b> 08/366,787                      30 December 1994 (30.12.94)      US			
<b>(71) Applicant:</b> CHIRON VIAGENE, INC. [US/US]; 4560 Horton Street, Emeryville, CA 94608 (US).			
<b>(72) Inventors:</b> DE POLO, Nicholas, J.; 567 Hygeia Avenue, Leucadia, CA 92024 (US). HSU, David, Chi-Tang; 8012 Camino Tranquila, San Diego, CA 92122 (US).			
<b>(74) Agents:</b> KRUSE, Norman, J. et al.; Chiron Corporation, Intellectual Property - R440, P.O. Box 8097, Emeryville, CA 94662-8097 (US).			
<b>(54) Title:</b> NUCLEIC ACID CONDENSING AGENTS WITH REDUCED IMMUNOGENICITY			
<b>(57) Abstract</b>  Nucleic acid condensing agents with reduced immunogenicity are generated either by conjugation of polycations or by selection of basic amino acid regions from proteins. Conjugation involves a chemical linkage between a polyalkylene glycol, such as polyethylene glycol, or a polysaccharide, such as dextran, and a polycation. Additionally, gene delivery vehicles, such as viral vectors, may be conjugated with polyalkylene glycol or polysaccharide, to reduce their immunogenicity. Basic amino acid regions of proteins are identified by isoelectric point, and amino acid composition. These condensing agents are complexed with nucleic acids and used to deliver genes to cells. Immunogenicity is assessed by whether neutralizing antibody is induced and by whether a serum component inactivates the complexes.			





32. The nucleic acids of claim 27, further comprising a ligand capable of targeting the nucleic acids to a selected cell type.

33. The nucleic acids of claim 32 wherein the ligand is selected from the group consisting of transferring, asialoglycoprotein, antibody, antibody fragments, low density lipoproteins, interleukins, GM-CSF, G-CSF, M-CSF, stem cell factor and erythropoietin.

34. A method of gene transfer in a patient comprising administering the nucleic acids according to any one of claims 1, 8, 14, 20, or 25 in a pharmaceutically acceptable carrier to a patient.

35. The method according to claim 34, further comprising adding a ligand to the nucleic acid condensing agent which is capable of targeting the nucleic acids to a selected cell type.

36. The method according to claim 34 wherein the nucleic acids stimulate an immune response upon administration to the patient.

37. The method according to claim 34 wherein the nucleic acids suppress an immune response.

38. The method according to claim 34 wherein the nucleic acids encode a prodrug.

39. The method according to claim 34 wherein the nucleic acids encode a cytokine.

40. The method according to claim 34 wherein the nucleic acids are administered to a tumor.

41. The method according to claim 34 wherein the nucleic acids are capable of constitutive production of protein.

22. The gene delivery vehicle of claim 21 wherein the polysaccharide is dextran.

23. The gene delivery vehicle of claim 22 wherein the dextran has a molecular weight ranging from 1,000 to 90,000.

24. The gene delivery vehicle of claim 22, further comprising a pharmaceutically acceptable carrier.

25. The gene delivery vehicle of claim 22, further comprising a ligand capable of targeting the nucleic acids to a selected cell type.

26. The gene delivery vehicle of claim 25 wherein the ligand is selected from the group consisting of transferrin, asialoglycoprotein, antibody, antibody fragments, low density lipoproteins, interleukins, GM-CSF, G-CSF, M-CSF, stem cell factor and erythropoietin.

27. Nucleic acids condensed with a nucleic acid condensing agent comprising a basic amino acid region of at least 50 amino acids, wherein the region has at least 40% basic amino acids, less than 5% acidic amino acids and a predicted isoelectric point of at least 9, wherein the nucleic acid condensing agent in combination with nucleic acids exhibits low immunogenicity.

28. The nucleic acids of claim 27 wherein the basic amino acid region is derived from a protein selected from the group consisting of histones and protamines.

29. The nucleic acids of claim 27 wherein there are a plurality of basic amino acid regions in a tandem array.

30. The nucleic acids of claim 29 wherein the array contains 1-10 basic amino acid regions.

31. The nucleic acids of claim 27, further comprising a pharmaceutically acceptable carrier.

11. The nucleic acids of claim 8 wherein the polycation is selected from the group consisting of polylysine, protamines, spermine, spermidine, polyornithine, polyarginine and putrescine.
12. The nucleic acids of claim 8, further comprising a pharmaceutically acceptable carrier.
13. The nucleic acids of claim 8, further comprising a ligand capable of targeting the nucleic acids to a selected cell type.
14. The nucleic acids of claim 13 wherein the ligand is selected from the group consisting of transferrin, asialoglycoprotein, antibody, antibody fragments, low density lipoproteins, interleukins, GM-CSF, G-CSF, M-CSF, stem cell factor and erythropoietin.
15. A gene delivery vehicle chemically conjugated with polyalkylene glycol, the conjugated product exhibiting low immunogenicity.
16. The gene delivery vehicle of claim 15 wherein the polyalkylene glycol is polyethylene glycol.
17. The gene delivery vehicle of claim 16 wherein the polyethylene glycol has a molecular weight ranging from 200 to 10,000.
18. The gene delivery vehicle of claim 15, further comprising a pharmaceutically acceptable carrier.
19. The gene delivery vehicle of claim 15, further comprising a ligand capable of targeting the gene delivery vehicle to a selected cell type.
20. The gene delivery vehicle of claim 19 wherein the ligand is selected from the group consisting of transferrin, asialoglycoprotein, antibody, antibody fragments, low density lipoproteins, interleukins, GM-CSF, G-CSF, M-CSF, stem cell factor, and erythropoietin.
21. A gene delivery vehicle chemically conjugated with polysaccharide, the conjugated product exhibiting low immunogenicity.

Claims

1. Nucleic acids condensed with a nucleic acid condensing agent comprising a polycation chemically conjugated with polyalkylene glycol, wherein the nucleic acid condensing agent in combination with nucleic acids exhibits low immunogenicity.
2. The nucleic acids of claim 1 wherein the polyalkylene glycol is polyethylene glycol.
3. The nucleic acids of claim 2 wherein the polyethylene glycol has a molecular weight ranging from 200 to 10,000.
4. The nucleic acids of claim 1 wherein the polycation is selected from the group consisting of polylysine, protamines, spermine, spermidine, polyornithine, polyarginine and putrescine.
5. The nucleic acids of claim 1, further comprising a pharmaceutically acceptable carrier.
6. The nucleic acids of claim 1, further comprising a ligand capable of targeting the nucleic acids to a selected cell type.
7. The nucleic acids of claim 6 wherein the ligand is selected from the group consisting of transferrin, asialoglycoprotein, antibody, antibody fragments, low density lipoproteins, interleukins, GM-CSF, G-CSF, M-CSF, stem cell factor and erythropoietin.
8. Nucleic acids condensed with a nucleic acid condensing agent comprising a polycation chemically conjugated with a polysaccharide, wherein the nucleic acid condensing agent in combination with nucleic acids exhibits low immunogenicity.
9. The nucleic acids of claim 8 wherein the polysaccharide is dextran.
10. The nucleic acids of claim 9 wherein the dextran has a molecular weight ranging from 1,000 to 90,000.

give efficient gene transfer as described in Example 4. The complex is treated with heat-inactivated FBS (as a control), human sera, or heat-inactivated human sera. Transfection efficiency is then tested on a tissue culture target cell such as HT1080 human fibroblasts (ATCC No. CCL121).

- 5                   The assay for serum inactivation of the complex is performed as follows. Blood is drawn from at least two human volunteers. Approximately 20-70 mL of blood is collected and allowed to clot for 20-30 minutes at room temperature. Blood samples are centrifuged at 2000 x g for 10 minutes at 4°C. Serum is frozen in approximately 1.0 mL aliquots and stored at -80°C. Vials from each batch were tested for total classical
- 10 complement activity (Quantiplate™, Kallestad Labs, Inc., Chaska, MN), and only batches with normal (CH<sub>100</sub> unit activity at least 60) levels of complement activity were used. Heat-inactivated serum samples are prepared by heating for 30 minutes at 56°C. Quantities of β-gal plasmid DNA are condensed with the condensing agent such that 10<sup>4</sup> to 10<sup>5</sup> BCFU/ml are generated following a standard transfection onto HT1080 cells). Complexes
- 15 are mixed 1:10 with heat-inactivated FBS, human sera, or human heat-inactivated sera and incubated at 37°C for 30 minutes. Transfection efficiency is determined by a standard BCFU assay (*Current Protocols in Molecular Biology, supra*). If a human serum component inactivates the complex, the number of BCFU is reduced relative to treatment with heat-inactivated FBS (control). If the serum component is heat-labile, the number of
- 20 BCFU following treatment with heat-inactivated human sera will not deviate significantly from the control. A condensing agent with reduced sensitivity to human serum inactivation is defined as an increase of BCFU of two-fold or greater relative to polylysine-condensed DNA.

- 25                   From the foregoing, it will be evident that although specific embodiments of the invention have been described herein for the purposes of illustrating the invention, various modifications may be made without deviating from the spirit and scope of the invention.

each tested nucleic acid complex. The conjugated polylysine-condensed DNA complex will meet the defined criteria for low immunogenicity if the antibody titer is reduced at least three fold and preferably ten fold relative to the titer determined for unconjugated polylysine/DNA complex at the two or the three week bleed time point.

5           In addition, if the criteria in the ELISA for low immunogenicity are not met, then these same sera that contain antibodies to the complex, either fresh or stored frozen at -80°C, are tested for neutralizing antibodies in the following assay. A reporter plasmid, such as pSV  $\beta$ -Galactosidase (Promega Cat. No. E1081), which expresses  $\beta$ -gal is condensed with modified or unmodified polylysine condensing agent under conditions that  
10       give efficient gene transfer as described in Example 4. The complex is treated with heat-inactivated FBS (as a control), or heat-inactivated mouse sera from the post injection bleeds of the modified or unmodified polylysine DNA complex injected mice respectively. Transfection efficiency is then tested on a tissue culture target cell such as HT1080 human fibroblasts (ATCC No. CCL121).

15           Heat-inactivated serum samples are prepared by heating for 30 minutes at 56°C. Quantities of  $\beta$ -gal plasmid DNA are reacted with the condensing agent such that approximately  $1 \times 10^5$  BCFU/ml are generated following a standard transfection onto HT1080 cells. Complexes are mixed 1:10 with heat-inactivated FBS, or heat-inactivated sera from mice previously injected with the same gene transfer complexes and incubated at  
20       37°C for 30 minutes. Transfection efficiency is determined by a standard BCFU assay (*Current Protocols in Molecular Biology, supra*). If the sera contains neutralizing antibodies, the number of BCFU is reduced relative to treatment with heat-inactivated FBS (control). If the serum component is heat-labile, the number of BCFU following treatment with heat-inactivated sera will not deviate significantly from the control. "Percentage  
25       survival" is the percentage of BCFU/ml obtained from transfection of nucleic acid/condensing agent complex following treatment with heat-inactivated sera isolated from immune animals (i.e., animals injected with the same nucleic acid/condensing agent) relative to the number of BCFU/ml obtained following treatment with heat-inactivated FBS treatment. The GL2/conjugated-polylysine complex will meet the criteria for low  
30       immunogenicity if its percentage survival is at least three-fold, and preferably ten-fold, higher than the percentage survival for the GL2/unconjugated-polylysine complex.

#### Example 6

##### COMPLEMENT INACTIVATION ASSAY

35

A reporter plasmid, such as pSV- $\beta$ -Galactosidase (Promega Cat. No. E1081), which expresses  $\beta$ -gal is condensed with condensing agent under conditions that

Example 5

## DETERMINATION OF IMMUNOGENICITY

5           The immunogenicity of modified polylysine and nucleic acid condensed complexes is measured in comparison to unmodified polylysine nucleic acid condensed complexes. These complexes are administered to mice, rabbits, or primates and the extent of the immune response directed against the injected complexes is measured (A) by ELISA and, if antibodies are present, (B) by neutralization of gene transfer efficiency in tissue  
10 culture. Experiments in this example are performed in mice owing to the simplicity and ease of this animal model.

          An appropriate dose of unmodified polylysine/DNA to achieve measurable antibody levels is first determined. Groups of three mice, in a common strain such as Balb/c, are injected with either 1, 5, 25, or 125 µg of reporter gene construct DNA, pGL2-  
15 control complexed with polylysine as in Example 4. Briefly, 20 µg of unmodified polylysine (as a control) are mixed per 6 µg of DNA. The reactions are incubated at room temperature for 30 minutes to allow condensation to occur. This mixture is diluted in HEPES/NaCl buffer. It may be rapidly concentrated in ultrafiltration units, Centriprep-10 or 30 (Amicon) if necessary. The mixture is then administered into an adult mouse by i.v.,  
20 i.p., s.c., or i.m. injection. A total of 3 or more injections are administered to each mouse, with two to four weeks between each injection. At 1, 2, 3, 4, and 8 weeks after the final injection, blood samples are collected from each mouse. From each individual mouse, antibody titers against polylysine are measured in a standard ELISA assay (Engvall E., *Meth. Enzymol.* 70:419-39, 1980). Briefly, polylysine/DNA complex is used to coat the  
25 plates, and dilutions of serum samples are then added. Plates are washed to remove unbound serum antibodies. An anti-mouse immunoglobulin antibody conjugated with a reporter molecule, such as horseradish peroxidase (Tago), is added to each well. A colorimetric determination of the titer of the anti-polylysine/DNA complex antibody is then established. The experiment to determine the effects of modifications on immunogenicity is  
30 performed at the lowest convenient doses of plasmid DNA/polylysine complex which in the majority of animals give a measurable antibody induction response by the third bleed.

          Groups of five mice are then injected with unmodified or modified polylysine/DNA complexes prepared as described above, at the immunogenically appropriate dose and injection schedule determined in the dose response experiment. Mice  
35 are bled weekly for three weeks. Sera are separated following clotting at room temperature and tested individually at 1:100 to 1:10,000 dilutions in an ELISA. Titers are determined as the reciprocal of the dilution capable of generating 50% of the maximum response of



(Kodak). The ability of the candidate agents to condense DNA is determined by the minimal amount of agent required to retard the mobility of the DNA fragments in the agarose gel. For example, 100 ng of polylysine is able to retard the mobility of the DNA fragments under these conditions. Any condensing agent that retards the mobility of the DNA fragments with less than 100 ng is considered a better DNA condensing reagent than polylysine. Any agents that condense the DNA fragments using less than 200 ng are chosen for further analysis in assays measuring gene transfer efficiency.

Alternatively, 10  $\mu$ g of unlabeled pGL2 plasmid DNA in 200  $\mu$ l condensation buffer (20 mM Hepes/150 mM NaCl, pH 7.4) is mixed with various amounts of candidate condensing agents at similar ratios to above. The condensation reactions are incubated at room temperature for 30 minutes, at which time a fraction (1/10 of the total volume) of the samples are electrophoresed on a 0.8% agarose gel which is then stained with ethidium bromide (1  $\mu$ g/ml) for 30 minutes and washed with water for 30 minutes. The DNA bands are visualized on a transilluminator and photographed. Condensation of plasmid DNA is associated with a decrease in mobility during electrophoresis.

#### Example 4

##### EFFICIENCY OF GENE TRANSFER IN VITRO

Polylysine with an average length of 270 amino acid residues is used as a standard in a gene transfer assay. Condensing agents that mediate gene transfer with an efficiency of 50% that of polylysine are chosen for further analysis. Efficiency of polylysine-mediated gene transfer is determined as follows. 3  $\mu$ g of the luciferase reporter gene construct, pGL2-control, in 250  $\mu$ l of 20 mM Hepes/150 mM NaCl, pH 7.4 and 10  $\mu$ g polylysine (Sigma, Cat. #2636) in 250  $\mu$ l of 20 mM Hepes/150 mM NaCl, pH 7.4, are mixed and incubated at room temperature for 30 minutes, at which time 1 ml of DMEM/2% FBS (fetal bovine serum) is added. The mixture is added to target cells (e.g., HT1080, K562, or other primary cell lines), grown to about 60-80% confluency in 60 mm dishes. After 1 hour at 37°C, 4 ml of DMEM/10% FCS is added and incubation continued at 37°C in a CO<sub>2</sub> incubator for 24 hours. At this time, cells are harvested by scraping the dishes. Reagents for the luciferase assay are commercially available (e.g., Promega Corporation, part No. E1500). Procedures suggested by the manufacturer are followed for the assay. Briefly, harvested cells are lysed. The lysate is then mixed with Luciferase Assay Reagent containing luciferin, ATP, CoA, DTT, EDTA, MgSO<sub>4</sub>, and Tricine. Luciferase present in the lysate catalyzes a reaction that leads to emission of light. The efficiency of gene transfer is gauged by measuring the intensity of light emission from the mixture with a luminometer.

MW of specific polycation - nucleic acid

$$\frac{\text{complex}}{6,000} \times \frac{[\text{Dextran}] \text{ (mg/ml)}}{[\text{Polycation}] \text{ (mg/ml)}}$$

### Example 3

#### ASSAYS FOR DNA CONDENSATION

5 A number of assays may be used to detect the condensation of the DNA. These assays are generally based on the resulting changes in the physical properties of DNA after condensation. For example, the ability of DNA to bind ethidium bromide is reduced, and the mobility of DNA molecules during electrophoresis in an agarose gel is retarded.

10 An ethidium bromide dye assay is performed by mixing 10 µg pGL2 control plasmid DNA (Promega Corporation, part No. E1611) in 200 µl condensation buffer (20 mM Hepes/150 mM NaCl, pH 7.4) with candidate condensing agents. Different amounts of candidate condensing agents are added (*e.g.*, 10, 50, 100, 200 or 500 µg). The condensation reactions are incubated at room temperature for 30 minutes, at which time ethidium bromide is added to each reaction to a final concentration of 1 µg/ml. After 15

15 min, reaction mixtures are transferred into polystyrene tubes or other UV-transparent material and placed on a 260 nm transilluminator. The fluorescent light emission from the DNA-ethidium bromide complex in each reaction mixture is recorded with a camera equipped with a UV filter. The ability of a condensing agent to condense DNA is inversely proportional to the intensity of the fluorescent in each reaction mixture.

20 A band shift assay is based on the size difference between condensed and non-condensed DNA. Mobility of condensed DNA in an electric field is reduced and can be monitored by agarose gel electrophoresis. The procedure according to Wagner et al. (*Proc. Natl. Acad. Sci. USA* 87:3410, 1990) may be followed. Briefly, lambda DNA is digested with *EcoRI* and *HindIII* restriction enzymes to generate DNA fragments with a

25 size ranging from 564bp to 21.2kb. These fragments are radioactively labeled by filling in the cohesive ends with [<sup>32</sup>P]dNTPs using polI (Klenow fragment) (Sambrook et al., *Molecular Cloning-A Laboratory Manual*, 1989). Radioactively labeled DNA fragments (35 ng) are mixed with 10, 20, 50, 75, 100, 200, 500 or 1000 ng of the candidate condensing agents in 10 mM Hepes/200 mM NaCl, pH 7.9 at a final volume of 12 µl.

30 After incubating at room temperature for 30 minutes, the samples are electrophoresed on a 1.0% agarose gel with 1X TAE electrophoresis buffer (40 mM Tris acetate/1 mM EDTA, pH 8.0). The gel is dried and autoradiographed for 3 hours at -80°C with XAR film

in 1 liter of 10 mM sodium borate buffer, containing 0.35 M  $\text{NaIO}_4$ , pH 3.0. After 24 h in the dark, the reaction is terminated by adding a 2-fold molar excess of ethylene glycol for an additional 2 h. The reaction mixture is dialyzed extensively against distilled water and lyophilized. Yields (w/w) of oxidized dextran are typically 45% of starting material. The relative aldehyde content of oxidized dextran may be determined by anthrone assay (Fagnani et al., *Cancer Res.* 50:3638, 1990). Additionally, oxidized dextran may be analyzed for integrity by HPLC.

A solution of oxidized dextran in 100 mM sodium phosphate buffer, pH 7.0, is added in stoichiometric molar ratios from about 1:500 to 1:1500 in two-fold decreasing molar increments to a solution of polylysine (5 mg/ml) in the same buffer. A solution of  $\text{NaBH}_3\text{CN}$  in the same buffer is then added in stoichiometric molar ratios of 1 to 5 relative to dextran, and the solution stirred at 22°C for 2 h. At the end of the incubation, dextran-modified polylysine is separated from unreacted dextran by gel filtration. The level of dextran substitution of polylysine may be determined with the anthrone assay as described below. Unreacted aldehyde groups on dextran bound to polylysine is then reduced by dropwise addition of  $\text{NaBH}_4$ , in 0.05 N NaOH at a molar ratio of 25 to 1 relative to the glucose content of dextrose (33 glucose subunits per  $M_r$  6000 dextran). After incubation for 24 h at 4°C, completion of the reaction may be established by anthrone determination. Dextran-modified polylysine is then extensively dialyzed against 100 mM sodium phosphate, pH 7.0, and stored in this buffer at 4°C.

To determine the extent of dextran conjugation, a standard curve is constructed by adding increasing amounts of activated dextran (10 to 100  $\mu\text{g}$ ) to 1 ml of sodium phosphate buffer, pH 7.0, in clean glass tubes in duplicate. To this volume, 3 ml of a freshly made anthrone solution (0.2% in 80%  $\text{H}_2\text{SO}_4$ ) are added rapidly and tubes are mixed on a vortex mixer. Tubes then are transferred immediately to a boiling water bath for 15 minutes, and then cooled in an ice water bath for an additional 15 minutes. Color development is determined by reading the absorbance at 625 nm. in a spectrophotometer. With this assay, non-oxidized dextran or oxidized dextran which has been reduced with  $\text{NaBH}_4$  does not produce any color. Fifty to 100  $\mu\text{g}$  of either unmodified polycation or polycation dextran adducts then are similarly tested, and their absorbance at 625 nm. is plotted against the standard curve prepared from the previous activated dextran standard spectrophotometric determinations. The amount of activated dextran bound to a polycation can be determined from the curve and the molar concentration then is calculated. The concentration of polycation can be determined easily by reading the absorbance at 280 nm. The molar substitution of dextran (m.w. 6,000) per mole of polycation then can be calculated according to the following formula:

Methoxypolyethylene glycol (PEG) *N*-hydroxysuccinimidyl-glutarate is prepared by first conjugating glutarate to the free hydroxy terminus of PEG and then conjugating an *N*-hydroxysuccinimide in two steps. Adding a glutarate group is done following the procedure of Joppich and Luisi (*Macromol. Chem.* 180:1381, 1979). Fifty g of PEG are dissolved in 80 ml of toluene and distilled. After cooling, the solution is reacted with glutaric anhydride (11.5 g) in dicycloethane (200 ml) and dry pyridine (5 ml). The mixture is refluxed under nitrogen gas for 3 days, filtered and the solvent evaporated. The residue is dissolved in 100 ml of water and washed twice with 50 ml of diethyl ether. PEG-glutarate is then extracted with two 50 ml chloroform washes, and the residual chloroform is evaporated. Approximately 38 g of PEG-glutarate is obtained.

Next, NHS is attached to the glutarate (Anderson et al., *J. Am. Chem. Soc.* 86:1839, 1964). PEG-glutarate is dissolved in 200 ml of dimethylformamide at 37°C. 0.94 g of *N*-hydroxysuccinimide and 1.7 g of dicyclohexylcarbodiimide in dry dimethylformamide are added and the mixture stirred vigorously in an ice bath, followed by stirring at room temperature for 24 h. Precooled benzene (100 ml) is added, and methoxypolyethylene glycol *N*-hydroxysuccinimidyl glutarate is precipitated by the dropwise addition of 200 ml of petroleum ether at 0°C. The precipitate is collected on a sintered glass filter. NHS-PEG is reprecipitated three times. NHS-PEG may be stored in a desiccator at -20°C until used.

NHS-PEG is conjugated to a free amino group of polylysine at ratios of 2-30 in 0.1 M PBS (pH 7.5). The solution is stirred for 30 min at room temperature, and any remaining activated ester is removed by reaction with excess  $\epsilon$ -aminocaproic acid for 5 min. Unbound NHS-PEG may be removed from the reaction mixture by molecular-exclusion chromatography on P-100 (Bio-Rad) in 0.1 M PBS (pH 7.5). The attachment of PEG to polylysine may be confirmed by SDS-PAGE. The degree of amino group modification in the nucleic acid condensing agent is determined by measuring the number of free amino groups with TNBS (Synder and Sobocinski, *Anal. Biochem.* 126:433, 1982) or by NMR spectroscopy against standard PEG preparations.

### Example 2

#### CHEMICAL MODIFICATION OF POLYCATION WITH DEXTRANS

Oxidized dextran, which refers to periodate-oxidized dextran molecules containing multiple aldehyde functions, is prepared by oxidation with NaIO<sub>4</sub> (Bobb, H., *Adv. Carbohydr. Chem.* 11:1, 1956). Briefly, 30 g of dextran, *M<sub>r</sub>* 6000  $\pm$  500, is dissolved

with reverse transcriptase using oligo dT or random primers. The single stranded cDNA may then be amplified by PCR (see U.S. Patent Nos. 4,683,202, 4,683,195 and 4,800,159. See also *PCR Technology: Principles and Applications for DNA Amplification*, Erlich (ed.), Stockton Press, 1989) utilizing oligonucleotide primers complementary to sequences on either side of desired sequences. In particular, a double stranded DNA is denatured by heating in the presence of heat stable Taq polymerase, sequence specific DNA primers, ATP, CTP, GTP and TTP. Double-stranded DNA is produced when synthesis is complete. This cycle may be repeated many times, resulting in a factorial amplification of the desired DNA.

10 Nucleic acid molecules that are suitable for use with the present invention may also be synthesized, for example, on an Applied Biosystems Inc. DNA synthesizer (e.g., APB DNA synthesizer model 392 (Foster City, California).

#### ADMINISTRATION

15 In aspects of the invention the nucleic acids condensed with a nucleic acid condensing agent are provided in a pharmaceutically acceptable carrier. Such compositions may comprise buffers such as physiologically-buffered saline, phosphate-buffered saline, and the like, carbohydrates, such as glucose, mannose, sucrose or mannitol, proteins, polypeptides or amino acids, such as glycine, antioxidants, chelating agents such as EDTA or glutathione, adjuvants (e.g., aluminum hydroxide) and preservatives. In addition, 20 pharmaceutical compositions of the present invention may also contain one or more additional active ingredients, such as, for example, cytokines like  $\beta$ -interferon.

Compositions of the present invention may be formulated for the manner of administration indicated, including for example, for oral, nasal, venous, intracranial, intraperitoneal, subcutaneous, or intramuscular administration.

25 Pharmaceutical compositions of the present invention may be administered in a manner appropriate to the disease to be treated (or prevented). The quantity and frequency of administration will be determined by such factors as the condition of the patient, and the type and severity of the patient's disease.

30 The following examples are offered by way of illustration and not by way of limitation.

#### EXAMPLES

35

##### Example 1

##### CHEMICAL MODIFICATION OF POLYCATION WITH PEG

No. 41000 (containing a G to T mutation in the 12th codon of ras), and ATCC No. 41049 (containing a G to A mutation in the 12th codon).

Alternatively, plasmids which encode normal cellular components may also be obtained from depositories such as the ATCC (*see, for example*, ATCC No. 41001 which contains a sequence which encodes the normal ras protein, ATCC No. 57103 which encodes abl, and ATCC Nos. 59120 or 59121 which encode the bcr locus) and mutated to form the altered cellular component. Methods for mutagenizing particular sites may readily be accomplished using methods known in the art (*see* Sambrook et al., *supra.*, 15.3 *et seq.*). In particular, point mutations of normal cellular components such as ras may readily be accomplished by site-directed mutagenesis of the particular codon, for example, codons 12, 13 or 61.

Other nucleic acid molecules that encode the above-described substances, as well as other nucleic acid molecules that are advantageous for use within the present invention, may be readily obtained from a variety of sources, including for example depositories such as the American Type Culture Collection (ATCC, Rockville, Maryland), or from commercial sources such as British Bio-Technology Limited (Cowley, Oxford England). Representative examples include BBG 12 (containing the GM-CSF gene coding for the mature protein of 127 amino acids), BBG 6 (which contains sequences encoding gamma interferon), ATCC No. 39656 (which contains sequences encoding TNF), ATCC No. 20663 (which contains sequences encoding alpha interferon), ATCC Nos. 31902, 31902 and 39517 (which contains sequences encoding beta interferon), ATCC No. 67024 (which contains a sequence which encodes Interleukin-1b), ATCC Nos. 39405, 39452, 39516, 39626 and 39673 (which contains sequences encoding Interleukin-2), ATCC Nos. 59399, 59398, and 67326 (which contain sequences encoding Interleukin-3), ATCC No. 57592 (which contains sequences encoding Interleukin-4), ATCC Nos. 59394 and 59395 (which contain sequences encoding Interleukin-5), and ATCC No. 67153 (which contains sequences encoding Interleukin-6).

Molecularly cloned genomes which encode the hepatitis B virus may be obtained from a variety of sources including, for example, the American Type Culture Collection (ATCC, Rockville, Maryland). For example, ATCC No. 45020 contains the total genomic DNA of hepatitis B (extracted from purified Dane particles) (*see* Figure 3 of Blum et al., *TIG* 5(5):154-158, 1989) in the Bam HI site of pBR322 (Moriarty et al., *Proc. Natl. Acad. Sci. USA* 78:2606-2610, 1981). (Note that correctable errors occur in the sequence of ATCC No. 45020.)

Alternatively, cDNA sequences for use with the present invention may be obtained from cells which express or contain the sequences. Briefly, within one embodiment mRNA from a cell which expresses the gene of interest is reverse transcribed

cells. In cancers, cells may respond inappropriately or not at all to signals from other cells or factors. In autoimmune disease, there is inappropriate recognition of "self" markers. Within the present invention, such interactions may be blocked by utilizing nucleic acids that produce, *in vivo*, an analogue to either of the partners in an interaction. Such an analogue is known as a blocking agent.

This blocking action may occur intracellularly, on the cell membrane, or extracellularly. The blocking action of a viral or, in particular, a retroviral nucleic acid carrying a gene for a blocking agent, can be mediated either from inside a susceptible cell or by secreting a version of the blocking protein to locally block the pathogenic interaction.

For example, in the case of HIV, the two agents of interaction are the gp 120/gp 41 envelope protein and the CD4 receptor molecule. Thus, an appropriate blocker would be a nucleic acid expressing either an HIV env analogue that blocks HIV entry without causing pathogenic effects, or a CD4 receptor analogue. The CD4 analogue would be secreted and would function to protect neighboring cells, while the gp 120/gp 41 is secreted or produced only intracellularly so as to protect only the vector-containing cell. It may be advantageous to add human immunoglobulin heavy chains or other components to CD4 in order to enhance stability or complement lysis. Delivery of a retroviral vector encoding such a hybrid-soluble CD4 to a host results in a continuous supply of a stable hybrid molecule.

Vector particles leading to expression of HIV env may also be constructed. It will be evident to one skilled in the art which portions are capable of blocking virus adsorption without overt pathogenic side effects (Willey et al., *J. Virol.* 62:139, 1988; Fisher et al., *Science* 233:655, 1986).

Another aspect of the invention involves the delivery of suppressor genes which, when deleted, mutated or not expressed in a cell type, lead to tumorigenesis in that cell type. Reintroduction of the deleted gene by means of a viral vector leads to regression of the tumor phenotype in these cells. Since malignancy can be considered to be an inhibition of cellular terminal differentiation compared with cell growth, the delivery and expression of gene products which lead to differentiation of a tumor should also, in general, lead to regression.

Sequences which encode the above-described altered cellular components may be obtained from a variety of sources. For example, plasmids which contain sequences that encode altered cellular products may be obtained from a depository such as the American Type Culture Collection (ATCC, Rockville, Maryland), or from commercial sources such as Advanced Biotechnologies (Columbia, Maryland). Representative examples of plasmids containing some of the above-described sequences include ATCC

More specifically, E3 encodes a 19 kD transmembrane glycoprotein, E3/19K, transcribed from the E3 region of the adenovirus 2 genome. Within the context of the present invention, tissue cells are transformed with a recombinant nucleic acid containing the E3/19K sequence, which upon expression produces the E3/19K protein. The E3/19K  
5 protein inhibits the surface expression of MHC class I surface molecules, and cells transformed by the nucleic acid evade an immune response. Consequently, donor cells can be transplanted with reduced risk of graft rejection and may require only a minimal immunosuppressive regimen for the transplant patient. This allows an acceptable donor-recipient chimeric state to exist with fewer complications. Similar treatments may be used  
10 to treat the range of so-called autoimmune diseases, including systemic lupus erythematosus, multiple sclerosis, rheumatoid arthritis or chronic hepatitis B infection.

Another alternative method of immunosuppression involves the use of antisense message, ribozyme, or other gene expression inhibitor specific for T-cell clones which are autoreactive in nature. These block the expression of the T-cell receptor of  
15 particular unwanted clones responsible for an autoimmune response. The anti-sense, ribozyme, or other gene may be introduced using a viral vector delivery system.

Other proteins, not discussed above, that function to inhibit, suppress or down-regulate MHC class I antigen presentation may also be identified and utilized within the context of the present invention. In order to identify such proteins, in particular those  
20 derived from mammalian pathogens (and, in turn, active portions thereof), a recombinant nucleic acid that expresses a protein or an active portion thereof suspected of being capable of inhibiting MHC class I antigen presentation is transformed into a tester cell line, such as BC. The tester cell lines with and without the sequence encoding the candidate protein are compared to stimulators and/or targets in the CTL assay. A decrease in cell lysis  
25 corresponding to the transformed tester cell indicates that the candidate protein is capable of inhibiting MHC presentation.

Another alternative method to determine down-regulation of MHC class I surface expression is by FACS analysis. More specifically, cell lines are transformed with a recombinant nucleic acid encoding the candidate protein. After drug selection and  
30 expansion, the cells are analyzed by FACS for MHC class I expression and compared to that of non-transformed cells. A decrease in cell surface expression of MHC class I indicates that the candidate protein is capable of inhibiting MHC presentation. This aspect of the present invention is further discussed in co-pending application Serial No. 08/116,827.

35 Many infectious diseases, cancers, autoimmune diseases, and other diseases involve the interaction of viral particles with cells, cells with cells, or cells with factors. In viral infections, viruses commonly enter cells via receptors on the surface of susceptible



by biochemical methods. For example, a monoclonal antibody that blocks the active portion of the protein can be used to isolate and purify the active portion of the cleaved protein (Harlow et al., *Antibodies: A Laboratory Manual*, Cold Springs Harbor, 1988).

Within one embodiment, the suppression is effected by specifically inhibiting  
5 the activation of display of processed peptides in the context of self MHC molecules along with accessory molecules such as CD8, intercellular adhesion molecule -1 (ICAM-1), ICAM-2, ICAM-3, leukocyte functional antigen-1 (LFA-1) (Altmann et al., *Nature* 338:521, 1989), the B7.1-3 molecule (Freeman et al., *J. Immunol.* 143:2714, 1989), LFA-3 (Singer, *Science* 255:1671, 1992; Rao, *Crit. Rev. Immunol.* 10:495, 1991), or other cell  
10 adhesion molecules. Antigenic peptide presentation in association with MHC class I molecules leads to CTL activation. Transfer and stable integration of specific sequences capable of expressing products expected to inhibit MHC antigen presentation block activation of T-cells, such as CD8<sup>+</sup> CTL, and therefore suppress graft rejection. A standard CTL assay is used to detect this response. Components of the antigen  
15 presentation pathway include the 45 Kd MHC class I heavy chain,  $\beta_2$ -microglobulin, processing enzymes such as proteases, accessory molecules, chaperonins such as calnexin (Gaczynska, et al., *Nature*, 365: 264-282, 1993), and transporter proteins such as PSF1, TAP1 and TAP 2 (Driscoll, et al., *Nature*, 365: 262-263, 1993).

In an alternative example, the recombinant nucleic acid directs the  
20 expression of a gene product or an active portion of a gene product capable of binding  $\beta_2$ -microglobulin. Transport of MHC class I molecules to the cell surface for antigen presentation requires association with  $\beta_2$ -microglobulin. Thus, proteins that bind  $\beta_2$ -microglobulin and inhibit its association with MHC class I indirectly inhibit MHC class I antigen presentation. Suitable proteins include the H301 gene product. Briefly, the H301  
25 gene, obtained from the human cytomegalovirus (CMV) encodes a glycoprotein with sequence homology to the  $\beta_2$ -microglobulin binding site on the heavy chain of the MHC class I molecule (Browne et al., *Nature* 347:770, 1990). H301 binds  $\beta_2$ -microglobulin, thereby preventing the maturation of MHC class I molecules, and renders transformed cells unrecognizable by cytotoxic T-cells, thus evading MHC class I restricted immune  
30 surveillance.

Within another embodiment, the recombinant nucleic acid directs the expression of a protein or active portion of a protein that binds to newly synthesized MHC class I molecules intracellularly. This binding prevents migration of the MHC class I molecule from the endoplasmic reticulum, resulting in the inhibition of terminal  
35 glycosylation. This blocks transport of these molecules to the cell surface and prevents cell recognition and lysis by CTL. For instance, one of the products of the E3 gene may be used to inhibit transport of MHC class I molecules to the surface of the transformed cell.

Transgenic animals, such as transgenic mice, may also be utilized to assess the tumorigenicity of an anti-tumor agent (e.g., Stewart et al., *Cell* 38:627-637, 1984; Quaife et al., *Cell* 48:1023-1034, 1987; and Koike et al., *Proc. Natl. Acad. Sci. USA* 86:5615-5619, 1989). In transgenic animals, the gene of interest may be expressed in all  
5 tissues of the animal. This unregulated expression of the transgene may serve as a model for the tumorigenic potential of the newly introduced gene.

In addition to tumorigenicity studies, it is generally preferable to determine the toxicity of the toxic palliatives, such as anti-tumor agent(s), prior to administration. A variety of methods well known to those of skill in the art may be utilized to measure such  
10 toxicity, including for example, clinical chemistry assays which measure the systemic levels of various proteins and enzymes, as well as blood cell volume and number.

Cell mediated and humoral responses may also be induced against a pathogenic agent, particularly viral and bacterial diseases, by administration of immunogenic portion(s) as discussed above. Briefly, immunogenic portions carrying  
15 relevant epitopes can be produced in a number of known ways (Ellis and Gerety, *J. Med. Virol.* 31:54-58, 1990), including chemical synthesis (Bergot et al., *Applied Biosystems Peptide Synthesizer User Bulletin No. 16*, 1986, Applied Biosystems, Foster City, California) and DNA expression in recombinant systems, such as the insect-derived baculovirus system (Doerfler, *Current Topics in Immunology* 131:51-68, 1986),  
20 mammalian-derived systems (such as CHO cells) (Berman et al., *J. Virol.* 63:3489-3498, 1989), yeast-derived systems (McAleer et al., *Nature* 307:178-180), and prokaryotic systems (Burrell et al., *Nature* 279:43-47, 1979).

The present invention also provides a nucleic acid capable of immune down-regulation. Specific down-regulation of inappropriate or unwanted immune responses,  
25 such as in autoimmune or pseudo-autoimmune diseases such as chronic hepatitis, diabetes, rheumatoid arthritis, graft vs. host disease and Alzheimer's, or in transplants of heterologous tissue such as bone marrow, can be engineered using immune-suppressive viral gene products, or active portion thereof, which suppress surface expression of transplantation (MHC) antigen. Within the present invention, an "active portion" of a gene  
30 product is that fragment of the gene product which must be retained for biological activity. Such fragments or active domains can be readily identified by systematically removing nucleotide sequences from the protein sequence, transforming target cells with the resulting recombinant nucleic acid, and determining MHC class I presentation on the surface of cells using FACS analysis or other immunological assays, such as a CTL assay. These fragments  
35 are particularly useful when the size of the sequence encoding the entire protein exceeds the capacity of the viral carrier. Alternatively, the active domain of the MHC antigen presentation inhibitor protein can be enzymatically digested and the active portion purified

tumorigenic. Representative examples of such altered cellular components which are not tumorigenic include Rb\*, ubiquitin\*, and mucin\*.

As noted above, in order to generate an appropriate immune response, the altered cellular component must also be immunogenic. Immunogenicity of a particular  
5 sequence is often difficult to predict, although T cell epitopes often possess an immunogenic amphipathic alpha-helix component. In general, however, it is preferable to determine immunogenicity in an assay. Representative assays include an ELISA which detects the presence of antibodies against the newly introduced vector, as well as assays which test for T helper cells such as gamma-interferon assays, IL-2 production assays, and  
10 proliferation assays. A particularly preferred method for determining immunogenicity is the CTL assay.

Once a sequence encoding at least one anti-tumor agent has been obtained, it is preferable to ensure that the sequence encodes a non-tumorigenic protein. Various assays are known and may easily be accomplished which assess the tumorigenicity of a  
15 particular cellular component. Representative assays include tumor formation in nude mice or rats, colony formation in soft agar, and preparation of transgenic animals, such as transgenic mice.

For this and many other aspects of the invention, tumor formation in nude mice or rats is a particularly important and sensitive method for determining the  
20 tumorigenicity of an anti-tumor agent. Nude mice lack a functional cellular immune system (*i.e.*, do not possess CTLs), and therefore provide a useful *in vivo* model in which to test the tumorigenic potential of cells. Normal non-tumorigenic cells do not display uncontrolled growth properties if injected into nude mice. However, transformed cells will rapidly proliferate and generate tumors in nude mice. Briefly, in one embodiment the  
25 nucleic acid is delivered to syngeneic murine cells, followed by administration into nude mice. The mice are visually examined for a period of 2 to 8 weeks after administration in order to determine tumor growth. The mice may also be sacrificed and autopsied in order to determine whether tumors are present. (Giovanela et al., *J. Natl. Cancer Inst.* 48:1531-1533, 1972; Furesz et al., "Tumorigenicity testing of cell lines considered for production of  
30 biological drugs," *Abnormal Cells, New Products and Risk*, Hopps and Petricciani (eds.), Tissue Culture Association, 1985; and Levenbook et al., *J. Biol. Std.* 13:135-141, 1985). Tumorigenicity may also be assessed by visualizing colony formation in soft agar (MacPherson and Montagnier, *Vir.* 23:291-294, 1964). Briefly, one property of normal non-tumorigenic cells is "contact inhibition" (*i.e.*, cells will stop proliferating when they  
35 touch neighboring cells). If cells are plated in a semi-solid agar support medium, normal cells rapidly become contact inhibited and stop proliferating, whereas tumorigenic cells will continue to proliferate and form colonies in soft agar.

encoded by these sequence(s) may be used as a marker of tumorigenic cells, and an immune response directed against these novel coding region(s) may be utilized to destroy tumorigenic cells containing the altered sequence(s) or gene(s).

5 If the altered cellular component is associated with making the cell tumorigenic, then, it is necessary to make the altered cellular component non-tumorigenic. For example, within one embodiment, the sequence or gene of interest which encodes the altered cellular component is truncated in order to render the gene product non-tumorigenic. The gene encoding the altered cellular component may be truncated to a variety of sizes, although it is preferable to retain as much as possible of the altered cellular  
10 component. In addition, it is necessary that any truncation leave intact at least some of the immunogenic sequences of the altered cellular component. Alternatively, multiple translational termination codons may be introduced into the gene which encodes the altered cellular component, downstream of the immunogenic region. Insertion of termination codons will prematurely terminate protein expression, thus preventing expression of the  
15 transforming portion of the protein.

Within one embodiment, the  $ras^*$  gene is truncated in order to render the  $ras^*$  protein non-tumorigenic. Briefly, the carboxy-terminal amino acids of  $ras^*$  functionally allow the protein to attach to the cell membrane. Truncation of these sequences renders the altered cellular component non-tumorigenic. Preferably, the  $ras^*$   
20 gene is truncated in the purine ring formation, for example around the sequence which encodes amino acid number 110. The  $ras^*$  gene sequence may be truncated such that as little as about 20 amino acids (including the altered amino acid(s) are encoded by the nucleic acid, although preferably, as many amino acids as possible should be expressed (while maintaining non-tumorigenicity).

25 Within another embodiment, the  $p53^*$  protein is modified by truncation in order to render the cellular component non-tumorigenic. As noted above, not all mutations of the  $p53$  protein are tumorigenic, and therefore, not all mutations would have to be truncated. Nevertheless, within a preferred embodiment,  $p53^*$  is truncated to a sequence which encodes amino acids 100 to 300, thereby including all four major "hot spots."

30 Other altered cellular components which are oncogenic may also be truncated in order to render them non-tumorigenic. For example, both *neu* and *bcr/abl* may be truncated in order to render them non-tumorigenic. Non-tumorigenicity may be confirmed by assaying the truncated altered cellular component as described above.

It should be noted, however, that if the altered cellular component is only  
35 associated with non-tumorigenic cells in general, and is not required or essential for making the cell tumorigenic, then it is not necessary to render the cellular component non-

cysteine mutation in a patient with mandibular osteoma, and (5) codon 713, a serine to stop mutation in another patient with mandibular osteoma (Nishiho et al., *Science* 253:665-669, 1991). In addition, six point mutations were identified in MCC codon numbers 12, 145, 267, 490, 506, and 698, as well as an additional 4 somatic mutations in APC (codons  
5 number 289, 332, 438, and 1338).

Within other embodiments of the invention, a nucleic acid is provided which directs the expression of an altered receptor which is functionally locked or stuck in an "ON" or "OFF" mode. Briefly, many cellular receptors are involved in cell growth by monitoring the external environment and signaling the cell to respond appropriately. If  
10 either the monitoring or signaling mechanisms fail, the cell will no longer respond to the external environment and may exhibit uncontrolled growth. Many different receptors or receptor-like structures may function as altered cellular components, including, for example, *neu* and mutated or altered forms of the thyroid hormone receptor, the PDGF receptor, the insulin receptor, the Interleukin receptors (e.g., IL-1, -2, -3, etc. receptors),  
15 or the CSF receptors, such as the G-CSF, GM-CSF, or M-CSF receptors.

For example, *neu* (also referred to as the Human Epidermal Growth Factor Receptor "HER" or the Epidermal Growth Factor "EGF" receptor) is an altered receptor which is found in at least 28% of women with breast cancer. A cDNA clone which encodes this protein has been isolated (Slamon et al., *Science* 244:707-712, 1989; Slamon  
20 et al., *Cancer Cells* 7:371-380, 1989; Shih et al., *Nature* 290:261, 1981). This clone encodes a protein that has extracellular, transmembrane, and intracellular domains (Schechter, *Nature* 312:513, 1984; Coussens et al., *Science* 230:1132, 1985) and thus is believed to encode the *neu* receptor.

Studies of the rat *neu* gene isolated from chemically induced  
25 neuroglioblastoma cells indicate that it contains a single mutation at position 664 from valine to glutamic acid (Bargmann et al., *EMBO J.* 7:2043, 1988). In other studies, baby rats which were treated with N-ethyl-N-nitrosourea developed malignant tumors of the nervous system. All 47 trigeminal schwannomas and 12 neurinomas which developed carried a T to A transversion at position 664 of the *neu* gene (Nikitin et al., *Proc. Natl.*  
30 *Acad. Sci USA* 88:9939-9943, 1991).

Other altered receptors may also be expressed by nucleic acids in order to destroy selected tumor cells. For example, a deletion in chromosome 3p21-p25 has been associated with small-cell lung carcinomas (Leduc et al., *Am. J. Hum. Genet.* 44:282-287, 1989). A deletion is believed to occur in the ERBAb gene which otherwise codes for a  
35 DNA-binding thyroid hormone receptor (THR).

Alterations in receptors as described above result in the production of protein(s) (or receptors) containing novel coding sequence(s). The novel protein(s)

*Cancer Res.* 51:2908-2916, 1991). CTL lines which have been developed to breast tumors which cross-react with pancreatic tumor targets, and further appear to specifically recognize the specific 20 amino-acid tandem repeat (Jerome et al., *supra*). A sequence encoding one or more of the 20 amino-acid tandem repeats may be expressed by a nucleic acid of the present invention, in order to develop an immune response against tumor cells which contain this sequence.

Within another embodiment of the present invention, a nucleic acid is provided which directs the expression of an altered DCC (deleted in colorectal carcinomas) gene. Briefly, a very common region of allelic loss in colorectal tumors is chromosome 18q, which is lost in more than 70% of carcinomas, and in almost 50% of late adenomas. A presumptive tumor suppressor gene (DCC) from this region has been identified (Fearon et al., 1990), which encodes a protein with significant homology to cell-surface adhesion molecules, such as neural cell-adhesion molecule (NCAM) and contactin (reviewed by Edelman in *Biochem* 27:3533-3543, 1988). This protein is believed to play a role in the development of colorectal tumors, perhaps through alterations in normal cell-cell and/or cell-extracellular matrix interactions.

The DCC gene is expressed in normal colonic mucosa, but its expression is reduced or absent in the majority of colorectal carcinomas (Solomon, *Nature* 343:412-414, 1990). This loss of expression has been associated in some cases with somatic mutations of the DCC gene. A contiguous stretch of DNA comprising 370 kb has been cloned which encodes an approximately 750 amino acid protein (Fearon et al., *Science* 247:49-56, 1990).

Within another embodiment of the present invention, a nucleic acid is provided which directs the expression of MCC (mutated in colorectal cancer) or APC. Both MCC and APC have been identified as tumor suppressor genes (Kinzler et al., *Science* 251:1366-1370, 1991) which undergo mutation in familial adenomatous polyposis (FAP). FAP is believed to be the most common autosomal dominant disease which leads to cancer, and it affects at least 1 in 5,000 individuals in the United States (Nishiho et al., *Science* 253:665-669, 1991). Affected individuals usually develop hundreds to thousands of adenomatous polyps of the colon and rectum, which may progress to carcinoma. Gardner's syndrome ("GS," a variant of FAP) presents desmoid tumors, osteomas, and other neoplasms together with multiple adenomas of the colon and rectum. This proliferation is believed to be induced by loss or inactivation of the familial adenomatous polyposis gene (and in particular, MCC and APC) which is found on chromosome 5q.

For example, in Nishiho et al. (*supra*), the following germ line mutations of the APC gene were found in FAP and GS patients: (1) Codon 280, a serine to stop mutation (in a patient with mandibular osteoma), (2) codon 302, an arginine to stop mutation in two separate patients, one with a desmoid tumor, (3) codon 414, an arginine to

deletion in exon 21 in a prostate cancer and bladder cancer cell line (Bookstein et al., *Science* 247:712-715, 1990; Horowitz et al., *Science* 243:937, 1989), a deletion of exon 16 in a small-cell carcinoma of the lung (Shew et al., *Cell Growth and Diff.* 1:17, 1990), and a deletion between exons 21 and 27 (Shew et al., *Proc. Natl. Acad. Sci. USA* 87:6, 1990).

- 5 Deletion of these exons results in the production of a protein containing a novel coding sequence at the junction of the deleted exons. This novel protein coding sequence may be used as a marker of tumorigenic cells, and an immune response directed against this novel coding region may eliminate tumorigenic cells containing the Rb exon deletion.

- Within another embodiment of the present invention, a nucleic acid is  
10 provided which directs the expression of an altered gene which causes Wilms' tumor. Briefly, Wilms' tumor is typically found in children younger than 16 years of age. One child in 10,000 will develop this tumor, which comprises approximately 5% of childhood cancers. The tumor usually presents itself as a large abdominal mass which is surrounded by a fibrous pseudocapsule. Approximately 7% of the tumors are multifocal in one kidney,  
15 and 5.4% are involved with both kidneys. The Wilms' tumor gene has been localized to chromosome 11p13, and a cDNA clone (wt1) has been isolated that is characteristic of a tumor suppressor gene (Call et al., *Cell* 60:509, 1990; Gessler et al., *Nature* 343:744, 1990; Rose et al., *Cell* 60:495, 1990; and Haber et al., *Cell* 61:1257, 1990). The wt1 gene encodes a protein which contains four zinc fingers and a glutamine and proline rich amino  
20 terminus. Such structures are believed to be associated with transcriptional and regulatory functions.

- Mutations of the Wilms' tumor gene include the insertion of lysine, threonine, and serine between the third and forth zinc fingers. A wt1 protein which contains such insertions does not bind to the EGR-1 site. A second alternative mutation  
25 results in the insertion of about 17 amino acids in the region immediately NH<sub>2</sub>-terminal to the zinc finger domain (Madden et al., *Science* 253:1550-1553, 1991; Call et al., *Cell* 60:509, 1990; Gessler et al., *Nature* 343:744, 1990; Rose et al., *Cell* 60:495, 1990; Haber et al., *Cell* 61:1257, 1990; and Buckler et al., *Mol. Cell. Biol.* 11:1707, 1991).

- Within another embodiment of the present invention, a nucleic acid is  
30 provided which directs the expression of an altered mucin. Mucins are large molecular weight glycoproteins which contain approximately 50% carbohydrate. Polymorphic epithelial mucin (PEM) is a tumor-associated mucin (Girling et al., *Int. J. Cancer* 43:1072-1076, 1989) which is found in the serum of cancer patients. The full-length cDNA sequence has been identified (Gendler et al., *J. Biol. Chem.* 265(25):15286-15293, 1990;  
35 Lan et al., *J. Biol. Chem.* 265(25):15294-15299, 1990; and Ligtenberg et al., *J. Biol. Chem.* 265:5573-5578, 1990). Breast tumors and pancreatic tumors both express a mucin with an identical core sequence, containing a 20 amino-acid tandem repeat (Jerome et al.,

1991; Chiba et al., *Oncogene* 5:1603-1610, 1990 (pathogenesis of early stage non-small cell lung cancer is associated with somatic mutations in the p53 gene between codons 132 to 283); Prosser et al., *Oncogene* 5:1573-1579, 1990 (mutations in the p53 gene coding for amino acids 126 through 224 were identified in primary breast cancer); Cheng and Hass,  
5 *Mol. Cell. Biol.* 10:5502-5509, 1990; Bartek et al., *Oncogene* 5:893-899, 1990; Rodrigues et al., *Proc. Natl. Acad. Sci. USA* 87:7555-7559, 1990; Menon et al., *Proc. Natl. Acad. Sci. USA* 87:5435-5439, 1990; Mulligan et al., *Proc. Natl. Acad. Sci. USA* 87:5863-5867, 1990; and Romano et al., *Oncogene* 4:1483-1488, 1990 (identification of a p53 mutation at codon 156 in human osteosarcoma derived cell line HOS-SL)).

10 Certain alterations of the p53 gene may be due to certain specific toxins. For example, Bressac et al. (*Nature* 350:429-431, 1991) describes specific G to T mutations in codon 249, in patients affected with hepatocellular carcinoma. One suggested causative agent of this mutation is aflatoxin B<sub>1</sub>, a liver carcinogen which is known to be a food contaminant in Africa.

15 Four regions of the gene that are particularly affected occur at residues 132-145, 171-179, 239-248, and 272-286. Three "hot spots" of particular interest occur at residues 175, 248 and 273 (Levine et al., *Nature* 351:453-456, 1991). These alterations as well as others which are described above result in the production of protein(s) which contain novel coding sequence(s). The novel proteins encoded by these sequences may be  
20 used as a marker of tumorigenic cells, and an immune response directed against these novel coding regions may be utilized to destroy tumorigenic cells containing the altered sequence (p53\*).

Within another embodiment of the present invention, a nucleic acid is provided which directs the expression of an altered Rb (Rb\*) gene. Briefly, retinoblastoma  
25 is a childhood eye cancer associated with the loss of a gene locus designated Rb, which is located in chromosome band 13q14. A gene from this region has been cloned which produces a nuclear phosphoprotein of about 110 kd (Friend et al., *Nature* 323:643, 1986; Lee et al., *Science* 235:1394, 1987; and Fung et al., *Science* 236:1657, 1987).

Rb is believed to be a negative regulator of cellular proliferation, and has a  
30 role in transcriptional control and cell-cycle regulation. Rb binds to at least seven proteins found in the nucleus, and in particular, appears to be involved with a cellular transcription factor which has been designated both E2F (Bagchi et al., *Cell* 62:659-669, 1990) and DRTF (Shivji and La Thangue, *Mol. Cell. Biol.* 11:1686-1695, 1991). Rb is believed to restrict cellular growth by sequestering a variety of nuclear proteins involved in cellular  
35 proliferation.

Deletions within the Rb gene have been detected which evidence that the Rb gene may be responsible for tumorigenicity. These deletions include, for example, a



the cell cycle and normal growth properties. The normal ras protein is a G-protein which binds GTP and has GTPase activity, and is involved in transmitting signals from the external milieu to the inside of the cell, thereby allowing a cell to respond to its environment. Ras\* genes on the other hand alter the normal growth regulation of neoplastic cells by uncoupling cellular behavior from the environment, thus leading to the uncontrolled proliferation of neoplastic cells. Mutation of the ras gene is believed to be an early event in carcinogenesis (Kumar et al., "Activation of ras Oncogenes Preceding the Onset of Neoplasia," *Science* 248:1101-1104, 1990), which, if treated early, may prevent tumorigenesis.

10                Ras\* genes occur in a wide variety of cancers, including for example, pancreatic, colon, and lung adenocarcinomas. However, the spectrum of mutations occurring in the ras\* genes found in a variety of cancers is quite limited. These mutations alter the GTPase activity of the ras protein by converting the normal on/off switch to a constitutive ON position. Tumorigenic mutations in ras\* occur primarily (*in vivo*) in only 3  
15                codons: 12, 13 and 61. Codon 12 mutations are the most prevalent in both human and animal tumors.

              Within another embodiment of the present invention, a nucleic acid is provided which directs the expression of an altered p53 (p53\*) gene. Briefly, p53 is a nuclear phosphoprotein which was originally discovered in extracts of transformed cells,  
20                and thus was initially classified as an oncogene (Linzer and Levine, *Cell* 17:43-52, 1979; Lane and Crawford, *Nature* 278:261-263, 1979). It was later discovered that the original p53 cDNA clones were mutant forms of p53 (Hinds et al., *J. Virol.* 63:739-746, 1989). It now appears that p53 is a tumor suppressor gene, which negatively regulates the cell cycle, and that mutation of this gene may lead to tumor formation. Of colon carcinomas that have  
25                been studied, 75%-80% show a loss of both p53 alleles, one through deletion, and the other through point mutation. Similar mutations are found in lung cancer, and in brain and breast tumors.

              The majority of p53 mutations (e.g., p53\*1, p53\*2, etc.) are clustered between amino-acid residues 130 to 290 (*see* Levine et al., *Nature* 351:453-456, 1991; *see*  
30                also the following references which describe specific mutations in more detail: Baker et al., *Science* 244:217-221, 1989; Nigro et al., *Nature* 342:705-708, 1989 (p53 mutations cluster at four "hot spots" which coincide with the four highly conserved regions of the genes and these mutations are observed in human brain, breast, lung and colon tumors); Vogelstein, *Nature* 348:681-682, 1990; Takahashi et al., *Science* 246:491-494, 1989; Iggo et al.,  
35                *Lancet* 335:675-679, 1990; James et al., *Proc. Natl. Acad. Sci. USA* 86:2858-2862, 1989; Mackay et al., *Lancet* 11:1384-1385, 1988; Kelman et al., *Blood* 74:2318-2324, 1989; Malkin et al., *Science* 250:1233-1238, 1990; Baker et al., *Cancer Res.* 50:7717-7722,

FeLV (*see* Geering et al., *Virology* 36:678-680, 1968; Hardy et al., *JAMA* 158:1060-1069, 1971; Hardy et al., *Science* 166:1019-1021, 1969). Within one embodiment of the invention, the nucleic acid directs the expression of at least one portion of a feline leukemia virus antigen selected from the group consisting of p15gag, p12gag, p27gag, p10gag, p14pol, p80pol, p46pol, gp70env, and p15env. Within a particularly preferred embodiment, the nucleic acid directs the expression of gp85env. Sequences which encode these antigens may be readily obtained given the disclosure provided herein (*see* Donahue et al., *J. Virol.* 62(3):722-731, 1988; Stewart et al., *J. Virol.* 58(3):825-834, 1986; Kumar et al., *J. Virol.* 63(5):2379-2384, 1989; Elder et al., *J. Virol.* 46(3):871-880, 1983; Berry et al., *J. Virol.* 62(10):3631-3641, 1988; Laprevotte et al., *J. Virol.* 50(3):884-894, 1984).

Feline immunodeficiency virus (FIV) has been classified as a retrovirus of the lentivirus subfamily, based upon the magnesium requirement for reverse transcriptase (RT) and the morphology of viral particles (*see* Pedersen et al., *Science* 235:790-793, 1987). The feline immunodeficiency virus is morphologically and antigenically distinct from other feline retroviruses, including feline leukemia virus, type C oncornavirus (RD-114), and feline syncytium-forming virus (FeSFV) (*see* Yamamoto et al., "Efficacy of experimental FIV vaccines, (Abstract), First International Conference of Feline Immunodeficiency Virus Researchers, University of California, Davis, CA, Sep. 4-7, 1991). Within one embodiment of the invention, the nucleic acid directs the expression of at least one immunogenic portion of a feline immunodeficiency virus antigen selected from the group consisting of p15gag, p24gag, p10gag, p13pol, p62pol, p15pol and p36pol. Within a particularly preferred embodiment, the nucleic acid directs the expression of gp68env, gp27env and rev. Within the context of the present invention, "rev" is understood to refer to the antigen corresponding to the rev open reading frame (*see*, Phillips et al., First International Conference, *supra*). Sequences which encode these antigens may be readily obtained by one of skill in the art given the disclosure provided herein (*see* Phillips et al., *J. Vir.* 64(10):4605-4613, 1990; Olmsted et al., *PNAS* 86:2448-2452, 1989; Talbott et al., *PNAS* 86:5743-5747, 1989).

Still other examples include a nucleic acid which directs the expression of a non-tumorigenic, altered ras (ras\*) gene. Briefly, the ras\* gene is an attractive target because it is causally linked to the neoplastic phenotype, and indeed may be necessary for the induction and maintenance of tumorigenesis in a wide variety of distinct cancers, such as pancreatic carcinoma, colon carcinoma and lung adenocarcinoma. In addition, ras\* genes are found in pre-neoplastic tumors, and therefore immune intervention therapy may be applied prior to detection of a malignant tumor.

Normal ras genes are non-tumorigenic and ubiquitous in all mammals. They are highly conserved in evolution and appear to play an important role in maintenance of

Further, more than one immunogenic portion (as well as immunomodulatory factors, if desired) may be incorporated into the nucleic acid. For example, within one embodiment a nucleic acid may be prepared which directs the co-expression of both an immunogenic portion of the hepatitis B antigen, as well as an immunogenic portion of the hepatitis C polypeptide. Such constructs may be administered in order to prevent or treat acute and chronic hepatitis infections of either type B or C. Similarly, within other embodiments, a nucleic acid may be prepared which directs the co-expression of both an immunogenic portion of the hepatitis B X antigen, as well as an immunogenic portion of the hepatitis C polypeptide. Such a construct may similarly be administered in order to treat hepatocellular carcinoma that is associated with either hepatitis B or C. In addition, because those individuals chronically infected with hepatitis B and C are at higher risk for developing hepatocellular carcinoma, such a vector may also be utilized as a prophylactic treatment for the disease.

Immunogenic portions may also be selected by other methods. For example, the HLA A2.1/K<sup>b</sup> transgenic mouse has been shown to be useful as a model for human T-cell recognition of viral antigens. Briefly, in the influenza and hepatitis B viral systems, the murine T-cell receptor repertoire recognizes the same antigenic determinants recognized by human T-cells. In both systems, the CTL response generated in the HLA A2.1/K<sup>b</sup> transgenic mouse is directed toward virtually the same epitope as those recognized by human CTLs of the HLA A2.1 haplotype (Vitiello et al., *J. Exp. Med.* 173:1007-1015, 1991; Vitiello et al., *Abstract of Molecular Biology of Hepatitis B Virus Symposium*, 1992).

Immunogenic proteins of the present invention may also be manipulated by a variety of methods known in the art, in order to render them more immunogenic. Representative examples of such methods include: adding amino acid sequences that correspond to T helper epitopes; promoting cellular uptake by adding hydrophobic residues; by forming particulate structures; or any combination of these (*see generally*, Hart, op. cit., Milich et al., *Proc. Natl. Acad. Sci. USA* 85:1610-1614, 1988; Willis, *Nature* 340:323-324, 1989; Griffiths et al., *J. Virol.* 65:450-456, 1991).

The present invention also includes compositions and methods for treating, as well as vaccines for preventing, various feline diseases, including for example feline leukemia virus ("FeLV") and feline immunodeficiency virus ("FIV") infections. This viruses are discussed more fully in co-pending Application Serial No. 07/948,358.

Feline leukemia virus (FeLV) is a retrovirus of the oncornavirus subfamily. FeLV is presently believed to exist in three subgroups - A, B or C - which are differentiated by their envelope antigens gp70 and p15E. FeLV is also comprised of a number of core antigens, including p15, p12, p27, and p10, which are highly conserved for all subgroups of

blooded animal a nucleic acid which directs the expression of an immunogenic portion of antigen X, such that an immune response is generated. Sequences which encode the HBxAg may readily be obtained by one of skill in the art given the disclosure provided herein. Briefly, within one embodiment of the present invention, a 642 bp *Nco* I-*Taq* I  
5 fragment is recovered from ATCC 45020, and inserted into nucleic acids as described above for other hepatitis B antigens.

Within another aspect of the present invention, methods are provided for destroying hepatitis C carcinoma cells comprising the step of administering to a warm-blooded animal a nucleic acid which directs the expression of an immunogenic portion of a  
10 hepatitis C antigen. Preferred immunogenic portion(s) of a hepatitis C antigen may be found in the polypeptide which contains the core antigen and the NS1-NS5 regions (Choo et al., *Proc. Natl. Acad. Sci. USA* 88:2451-2455, 1991). Particularly preferred immunogenic portions may be determined by a variety of methods. For example, as noted above preferred immunogenic portions may be predicted based upon amino acid sequence.  
15 Briefly, various computer programs which are known to those of ordinary skill in the art may be utilized to predict CTL epitopes. For example, CTL epitopes for the HLA A2.1 haplotype may be predicted utilizing the HLA A2.1 motif described by Falk et al. (*Nature* 351:290, 1991). Another method that may also be utilized to predict immunogenic portions is to determine which portion has the property of CTL induction in mice utilizing  
20 retroviral vectors (*see*, Warner et al., *AIDS Res. and Human Retroviruses* 7:645-655, 1991). As noted within Warner et al., CTL induction in mice may be utilized to predict cellular immunogenicity in humans. Preferred immunogenic portions may also be deduced by determining which fragments of the polypeptide antigen or peptides are capable of inducing lysis by autologous patient lymphocytes of target cells (*e.g.*, autologous EBV-  
25 transformed lymphocytes) expressing the fragments after vector transduction of the corresponding genes.

Preferred immunogenic portions may also be selected in the following manner. Briefly, blood samples from a patient with a target disease, such as HCV, are analyzed with antibodies to individual HCV polypeptide regions (*e.g.*, HCV core, E1,  
30 E2/SN1 and NS2-NS5 regions), in order to determine which antigenic fragments are present in the patient's serum. In patients treated with alpha interferon to give temporary remission, some antigenic determinants will disappear and be supplanted by endogenous antibodies to the antigen. Such antigens are useful as immunogenic portions within the context of the present invention (Hayata et al., *Hepatology* 13:1022-1028, 1991; Davis  
35 et al., *N. Eng. J. Med.* 321:1501-1506, 1989).

With respect to the treatment of HBV, particularly preferred immunogenic portions for incorporation into nucleic acids include HBeAg, HBcAg, and HBsAg.

combinations of antigens may be preferred for administration in particular geographic regions.

Also presented by HBV are pol ("HBV pol"), ORF 5, and ORF 6 antigens. Briefly, the polymerase open reading frame of HBV encodes reverse transcriptase activity found in virions and core-like particles in infected liver tissue. The polymerase protein consists of at least two domains: the amino terminal domain encodes the protein that primes reverse transcription, and the carboxyl terminal domain which encodes reverse transcriptase and RNase H activity. Immunogenic portions of HBV pol may be determined utilizing methods utilizing nucleic acids administered in order to generate an immune response within an animal, preferably a warm-blooded animal. Similarly, other HBV antigens such as ORF 5 and ORF 6, (Miller et al., *Hepatology* 9:322-327, 1989), may be expressed utilizing nucleic acids as described herein.

As noted above, at least one immunogenic portion of a hepatitis B antigen can be incorporated into a nucleic acid. The immunogenic portion(s) which are incorporated into the nucleic acid may be of varying length, although it is generally preferred that the portions be at least 9 amino acids long, and may include the entire antigen. Immunogenicity of a particular sequence is often difficult to predict, although T cell epitopes may be predicted utilizing the HLA A2.1 motif described by Falk et al. (*Nature* 351:290, 1991). From this analysis, peptides may be synthesized and used as targets in an *in vitro* cytotoxic assay. Other assays, however, may also be utilized, including, for example, ELISA which detects the presence of antibodies against the newly introduced vector, as well as assays which test for T helper cells, such as gamma-interferon assays, IL-2 production assays, and proliferation assays.

Within one embodiment of the present invention, at least one immunogenic portion of a hepatitis C antigen can be incorporated into a nucleic acid. Preferred immunogenic portion(s) of hepatitis C may be found in the C and NS3-NS4 regions since these regions are the most conserved among various types of hepatitis C virus (Houghton et al., *Hepatology* 14:381-388, 1991). Particularly preferred immunogenic portions may be determined by a variety of methods. For example, as noted above for the hepatitis B virus, identification of immunogenic portions of the polypeptide may be predicted based upon amino acid sequence. Briefly, various computer programs which are known to those of ordinary skill in the art may be utilized to predict CTL epitopes. For example, CTL epitopes for the HLA A2.1 haplotype may be predicted utilizing the HLA A2.1 motif described by Falk et al. (*Nature* 351:290, 1991). From this analysis, peptides are synthesized and used as targets in an *in vitro* cytotoxic assay.

Within another aspect of the present invention, methods are provided for destroying hepatitis B carcinoma cells comprising the step of administering to a warm-

functional activity of T cells requires two signals. One signal is provided by interaction of the antigen-specific T cell receptor with peptides which are bound to major histocompatibility complex (MHC) molecules, and the second signal, referred to as costimulation, is delivered to the T cell by antigen presenting cells. The second signal is required for interleukin-2 (IL-2) production by T cells, and appears to involve interaction of the B7.1-.3 molecule on antigen-presenting cells with CD28 and CTLA-4 receptors on T lymphocytes (Linsley et al., *J. Exp. Med.*, 173:721-730, 1991a and *J. Exp. Med.*, 174:561-570, 1991). Within one embodiment of the invention, B7.1-.3 may be introduced into tumor cells in order to cause costimulation of CD8<sup>+</sup> T cells, such that the CD8<sup>+</sup> T cells produce enough IL-2 to expand and become fully activated. These CD8<sup>+</sup> T cells can kill tumor cells that are not expressing B7 because costimulation is no longer required for further CTL function. Vectors that express both the costimulatory B7.1-.3 factor, and, for example, an immunogenic HBV core protein, may be made utilizing methods which are described herein. Cells transduced with these vectors will become more effective antigen presenting cells. The HBV core-specific CTL response will be augmented from the fully activated CD8<sup>+</sup> T cell via the costimulatory ligand B7.1-.3.

The choice of which immunomodulatory factor to include within a nucleic acid may be based upon known therapeutic effects of the factor, or, experimentally determined. For example, a known therapeutic effector in chronic hepatitis B infections is alpha interferon. This has been found to be efficacious in compensating a patient's immunological deficit, and thereby assisting recovery from the disease. Alternatively, a suitable immunomodulatory factor may be experimentally determined. Briefly, blood samples are first taken from patients with a hepatic disease. Peripheral blood lymphocytes (PBLs) are restimulated *in vitro* with autologous or HLA matched cells (e.g., EBV transformed cells) that have been transduced with a nucleic acid which directs the expression of an immunogenic portion of a hepatitis antigen and the immunomodulatory factor. These stimulated PBLs are then used as effectors in a CTL assay with the HLA matched transduced cells as targets. An increase in CTL response over that seen in the same assay performed using HLA matched stimulator and target cells transduced with a vector encoding the antigen alone, indicates a useful immunomodulatory factor. Within one embodiment of the invention, the immunomodulatory factor gamma interferon is particularly preferred.

The present invention also includes immunogenic portions of desired antigens. For example, various immunogenic portions of the HBV S antigens may be combined in order to present an immune response when administered by one of the nucleic acids described herein. In addition, due to the large immunological variability that is found in different geographic regions for the S antigen open reading frame of HBV, particular

which are set forth above. Immunomodulatory factors refer to factors that, when manufactured by one or more of the cells involved in an immune response, or, which when added exogenously to the cells, causes the immune response to be different in quality or potency from that which would have occurred in the absence of the factors. The factors  
5 may also be expressed from a non-nucleic acid derived gene, but the expression is driven or controlled by the nucleic acid. The quality or potency of a response may be measured by a variety of assays known to one of skill in the art including, for example, *in vitro* assays which measure cellular proliferation (e.g.,  $^3\text{H}$  thymidine uptake), and *in vitro* cytotoxic assays (e.g., which measure  $^{51}\text{Cr}$  release) (see, Warner et al., *AIDS Res. and Human*  
10 *Retroviruses* 7:645-655, 1991). Immunomodulatory factors may be active both *in vivo* and *ex vivo*.

Representative examples of such factors include cytokines, such as IL-1, IL-2 (Karupiah et al., *J. Immunology* 144:290-298, 1990; Weber et al., *J. Exp. Med.* 166:1716-1733, 1987; Gansbacher et al., *J. Exp. Med.* 172:1217-1224, 1990; U.S. Patent  
15 No. 4,738,927), IL-3, IL-4 (Tepper et al., *Cell* 57:503-512, 1989; Golumbek et al., *Science* 254:713-716, 1991; U.S. Patent No. 5,017,691), IL-5, IL-6 (Brakenhof et al., *J. Immunol.* 139:4116-4121, 1987; WO 90/06370), IL-7 (U.S. Patent No. 4,965,195), IL-8, IL-9, IL-10, IL-11, IL-12, IL-13 (*Cytokine Bulliten*, Summer 1994), particularly IL-2, IL-4, IL-6, IL-12, and IL-13, alpha interferon (Finter et al., *Drugs* 42(5):749-765, 1991; U.S. Patent  
20 No. 4,892,743; U.S. Patent No. 4,966,843; WO 85/02862; Nagata et al., *Nature* 284:316-320, 1980; Familletti et al., *Methods in Enz.* 78:387-394, 1981; Twu et al., *Proc. Natl. Acad. Sci. USA* 86:2046-2050, 1989; Faktor et al., *Oncogene* 5:867-872, 1990), beta interferon (Seif et al., *J. Virol.* 65:664-671, 1991), gamma interferons (Radford et al., *The American Society of Hepatology* 20082015, 1991; Watanabe et al., *PNAS* 86:9456-9460,  
25 1989; Gansbacher et al., *Cancer Research* 50:7820-7825, 1990; Maio et al., *Can. Immunol. Immunother.* 30:34-42, 1989; U.S. Patent No. 4,762,791; U.S. Patent No. 4,727,138), G-CSF (U.S. Patent Nos. 4,999,291 and 4,810,643), GM-CSF (WO 85/04188), tumor necrosis factors (TNFs) (Jayaraman et al., *J. Immunology* 144:942-951, 1990), CD3 (Krissanen et al., *Immunogenetics* 26:258-266, 1987), ICAM-1 (Altman et al.,  
30 *Nature* 338:512-514, 1989; Simmons et al., *Nature* 331:624-627, 1988), ICAM-2, LFA-1, LFA-3 (Wallner et al., *J. Exp. Med.* 166(4):923-932, 1987), MHC class I molecules, MHC class II molecules, B7.1-3,  $\beta_2$ -microglobulin (Parnes et al., *PNAS* 78:2253-2257, 1981), chaperone-like molecules, such as calnexin, MHC-linked transporter proteins or analogs thereof (Powis et al., *Nature* 354:528-531, 1991). Within one preferred embodiment, the  
35 gene encodes gamma-interferon.

An example of an immunomodulatory factor cited above is a member of the B7 family of molecules (e.g., B7.1-3 costimulatory factor). Briefly, activation of the full

epitopes which may otherwise be overshadowed by immunodominant epitopes. Such an approach may be extended to the expression of a peptide having multiple epitopes, one or more of the epitopes being derived from different proteins. Further, this aspect of the invention allows efficient stimulation of cytotoxic T lymphocytes (CTL) directed against  
5 antigenic epitopes, and peptide fragments of antigens encoded by sub-fragments of genes, through intracellular generation and association of these peptide fragments with MHC class I molecules. This approach may be utilized to map major immunodominant epitopes for CTL induction.

An immune response can also be achieved by transferring to an appropriate  
10 immune cell (such as a B or T lymphocyte) (a) the gene for the specific T-cell receptor that recognizes the antigen of interest (in the context of an appropriate MHC molecule if necessary), (b) the gene for an immunoglobulin which recognizes the antigen of interest, or (c) the gene for a hybrid of the two which provides a CTL response in the absence of the MHC context. Thus the nucleic acid may be used as an immunostimulant,  
15 immunomodulator, or vaccine, etc.

In the particular case of disease caused by HIV infection, where immunostimulation is desired, the antigen generated from the recombinant retroviral genome is of a form which will elicit either or both an HLA class I- or class II-restricted immune response. In the case of HIV envelope antigen, for example, the antigen is  
20 preferably selected from gp 160, gp 120, and gp 41, which have been modified to reduce their pathogenicity. In particular, the selected antigen is modified to reduce the possibility of syncytia, to avoid expression of epitopes leading to a disease enhancing immune response, to remove immunodominant, but haplotype-specific epitopes or to present several haplotype-specific epitopes, and allow a response capable of eliminating cells infected with  
25 most or all strains of HIV. The haplotype-specific epitopes can be further selected to promote the stimulation of an immune response within an animal which is cross-reactive against other strains of HIV. Antigens from other HIV genes or combinations of genes, such as *gag*, *pol*, *rev*, *vif*, *nef*, *prot*, *gag/pol*, *gag prot*, etc., may also provide protection in particular cases.

HIV is only one example. This approach should be effective against many  
30 virally linked diseases or cancers where a characteristic antigen (which does not need to be a membrane protein) is expressed, such as in HPV and cervical carcinoma, HTLV-I-induced leukemias, prostate-specific antigen (PSA) and prostate cancer, mutated p53 and colon carcinoma and melanoma, melanoma specific antigens (MAGEs), and melanoma,  
35 mucin and breast cancer.

In accordance with the immunostimulation aspects of the invention, the substances of the present invention may also include "immunomodulatory factors," many of



applicable to monocytes and macrophages, which act as a reservoir for virus production as a result of their relative refractility to HIV-induced cytotoxicity (which, in turn, is apparently due to the relative lack of CD4 on their cell surfaces).

5                Still further aspects of the present invention relate to the administration of a nucleic acid capable of immunostimulation. The ability to recognize and defend against foreign pathogens is essential to the function of the immune system. In particular, the immune system must be capable of distinguishing "self" from "nonself" (*i.e.*, foreign), so that the defensive mechanisms of the host are directed toward invading entities instead of  
10              against host tissues. Cytolytic T lymphocytes (CTLs) are typically induced, or stimulated, by the display of a cell surface recognition structure, such as a processed, pathogen-specific peptide, in conjunction with a MHC class I or class II cell surface protein.

                 Diseases suitable to treatment include viral infections such as HIV, HBV and HPV, cancers such as melanomas, renal carcinoma, breast cancer, ovarian cancer and  
15              other cancers, and heart disease.

                 In one embodiment, the invention provides methods for stimulating a specific immune response and inhibiting viral spread by using nucleic acids that direct the expression of an antigen or modified form thereof in susceptible target cells, wherein the antigen is capable of either (1) initiating an immune response to the viral antigen or (2)  
20              preventing the viral spread by occupying cellular receptors required for viral interactions. Expression of the protein may be transient or stable with time. Where an immune response is to be stimulated to a pathogenic antigen, the recombinant viral vector is preferably designed to express a modified form of the antigen which will stimulate an immune response and which has reduced pathogenicity relative to the native antigen. This immune  
25              response is achieved when cells present antigens in the correct manner, *i.e.*, in the context of the MHC class I and/or II molecules along with accessory molecules such as CD3, ICAM-1, ICAM-2, LFA-1, or analogs thereof (*e.g.*, Altmann et al., *Nature* 338:512, 1989). In accordance with a preferred embodiment, cells infected with Sindbis viral vectors are expected to do this efficiently because they closely mimic genuine viral infection and (a) are  
30              able to infect non-replicating cells; (b) do not integrate into the host cell genome; and (c) are not associated with any life threatening diseases.

                 This embodiment of the invention has a further advantage over other systems that might be expected to function in a similar manner, in that the presenter cells are fully viable and healthy, and no other viral antigens (which may well be  
35              immunodominant) are expressed. This presents a distinct advantage since the antigenic epitopes expressed can be altered by selective cloning of sub-fragments of the gene for the antigen into the recombinant Sindbis virus, leading to responses against immunogenic

pathogenicity. Alternatively, the biologically active nucleic acid molecule may be a sense RNA (or DNA) complementary to RNA sequences necessary for pathogenicity.

More particularly, the biologically active nucleic acid molecule may be an antisense sequence. Briefly, antisense sequences are designed to bind to RNA transcripts, and thereby prevent cellular synthesis of a particular protein, or prevent use of that RNA sequence by the cell. Representative examples of such sequences include antisense thymidine kinase, antisense dihydrofolate reductase (Maher and Dolnick, *Arch. Biochem. & Biophys.* 253:214-220, 1987; Bzik et al., *PNAS* 84:8360-8364, 1987), antisense HER2 (Coussens et al., *Science* 230:1132-1139, 1985), antisense abl (Fainstein et al., *Oncogene* 4:1477-1481, 1989), antisense myc (Stanton et al., *Nature* 310:423-425, 1984) and antisense *ras*, as well as antisense sequences which block any of the enzymes in the nucleotide biosynthetic pathway.

In addition, within a further embodiment of the invention antisense RNA may be utilized as an anti-tumor agent in order to induce a potent class I restricted response. Briefly, in addition to binding RNA and thereby preventing translation of a specific mRNA, high levels of specific antisense sequences are believed to induce the increased expression of interferons (including gamma-interferon), due to the formation of large quantities of double-stranded RNA. The increased expression of gamma interferon, in turn, boosts the expression of MHC Class I antigens. Preferred antisense sequences for use in this regard include actin RNA, myosin RNA, and histone RNA. Antisense RNA which forms a mismatch with actin RNA is particularly preferred.

In another embodiment, the substances of the invention include a surface protein that is itself therapeutically beneficial. For example, in the particular case of HIV, expression of the human CD4 protein specifically in HIV-infected cells may be beneficial in two ways:

1. Binding of CD4 to HIV env intracellularly could inhibit the formation of viable viral particles much as soluble CD4 has been shown to do for free virus, but without the problem of systematic clearance and possible immunogenicity, since the protein will remain membrane bound and is structurally identical to endogenous CD4 (to which the patient should be immunologically tolerant).

2. Since the CD4/HIV env complex has been implicated as a cause of cell death, additional expression of CD4 (in the presence of excess HIV-env present in HIV-infected cells) leads to more rapid cell death and thus inhibits viral dissemination. This may be particularly

are regions of a protein that bind the protein to a membrane. Customarily, there are two types of anchor sequences that attach a protein to the outer surface of a cell membrane: (1) transmembrane regions that span the lipid bilayer of the cell membrane, and interact with the hydrophobic center region (proteins containing such regions are referred to integral membrane proteins), and (2) domains which interact with an integral membrane protein or with the polar surface of the membrane (such proteins are referred to as peripheral, or extrinsic, proteins).

Membrane anchors for use within the present invention may contain transmembrane domains which span the membrane one or more times. For example, in glycoporphin and guanylyl cyclase, the membrane binding region spans the membrane once, whereas the transmembrane domain of rhodopsin spans the membrane seven times, and that of the photosynthetic reaction center of *Rhodospseudomonas viridis* spans the membrane eleven times (see Ross et al., *J. Biol. Chem.* 257:4152, 1982; Garbers, *Pharmac. Ther.* 50:337-345, 1991; Engelman et al., *Proc. Natl. Acad. Sci. USA* 77:2023, 1980; Heijne and Manoil, *Prot. Eng.* 4:109-112, 1990). Particularly preferred membrane anchors for use within the present invention include naturally occurring cellular proteins (that are non-immunogenic) which have been demonstrated to function as membrane signal anchors (such as glycoporphin).

Within a preferred embodiment of the present invention, a DNA sequence is provided which encodes a membrane anchor - gamma interferon fusion protein. Within one embodiment, this fusion protein may be constructed by genetically fusing the sequence which encodes the membrane anchor of the gamma-chain of the Fc receptor, to a sequence which encodes gamma-interferon.

In yet another aspect, the nucleic acid provides a therapeutic effect by encoding one or more ribozymes (RNA enzymes) (Haseloff and Gerlach, *Nature* 334:585, 1989) which will cleave, and hence inactivate, RNA molecules corresponding to a pathogenic function. Since ribozymes function by recognizing a specific sequence in the target RNA and this sequence is normally 12 to 17 bp, this allows specific recognition of a particular RNA sequence corresponding to a pathogenic state, such as HIV tat, and toxicity is specific to such pathogenic state. Additional specificity may be achieved in some cases by making this a conditional toxic palliative, as discussed above.

In still another aspect, the nucleic acid comprises a biologically active nucleic acid molecule that is an antisense sequence (an antisense sequence may also be encoded by a nucleic acid sequence and then produced within a host cell via transcription). In preferred embodiments, the antisense sequence is selected from the group consisting of sequences which encode influenza virus, HIV, HSV, HPV, CMV, and HBV. The antisense sequence may also be an antisense RNA complementary to RNA sequences necessary for

infected with any particular virus. Additionally, since the human  $\alpha$  and  $\beta$  interferon promoter elements are transcriptionally activated in response to infection by a wide variety of nonrelated viruses, the introduction of vectors expressing a conditionally lethal gene product like HSVTK, for example, from these viral-responsive elements (VREs) could  
5 result in the destruction of cells infected with a variety of different viruses.

In another embodiment of the invention, methods are provided for producing substances such as inhibitor palliatives involving the delivery and expression of defective interfering viral structural proteins, which inhibit viral assembly. In this context, nucleic acid codes for defective gag, pol, env or other viral particle proteins or peptides  
10 which inhibit in a dominant fashion the assembly of viral particles. Such inhibition occurs because the interaction of normal subunits of the viral particle is disturbed by interaction with the defective subunits.

One way of increasing the effectiveness of inhibitory palliatives is to express inhibitory genes, such as viral inhibitory genes, in conjunction with the expression of genes  
15 which increase the probability of infection of the resistant cell by the virus in question. The result is a nonproductive "dead-end" event which would compete for productive infection events. In the specific case of HIV, a nucleic acid may be administered that inhibits HIV replication (by expressing anti-sense tat, etc., as described above) and also overexpress proteins required for infection, such as CD4. In this way, a relatively small number of  
20 vector-infected HIV-resistant cells act as a "sink" or "magnet" for multiple nonproductive fusion events with free virus or virally infected cells.

In another embodiment of the invention, methods are provided for the expression substances such as inhibiting peptides or proteins specific for viral protease. Viral protease cleaves the viral gag and gag/pol proteins into a number of smaller peptides  
25 Failure of this cleavage in all cases leads to complete inhibition of production of infectious retroviral particles. The HIV protease is known to be an aspartyl protease, and these are known to be inhibited by peptides made from amino acids from protein or analogues. Nucleic acids that inhibit HIV will express one or multiple fused copies of such peptide inhibitors.

30 The approaches discussed above should be effective against many virally linked diseases, cancers, or other pathogenic agents.

Within still other embodiments of the invention, a nucleic acid is provided that expresses a palliative, wherein the palliative has a membrane anchor and acts as an anti-tumor agent(s). Such a palliative may be constructed, for example, as an anti-tumor  
35 agent - membrane anchor fusion protein. Briefly, the membrane anchor aspect of the fusion protein may be selected from a variety of sequences, including, for example, the transmembrane domain of well known molecules. Generally, membrane anchor sequences

Within one embodiment of the invention, expression of a conditionally lethal HSVTK gene may be made even more HIV-specific by including cis-acting elements in the transcript ("CRS/CAR"), which require an additional HIV gene product, rev, for optimal activity (Rosen et al., *Proc. Natl. Acad. Sci. USA* 85:2071, 1988). More generally, cis elements present in mRNAs have been shown in some cases to regulate mRNA stability or translatability. Sequences of this type (*i.e.*, post-transcriptional regulation of gene expression) may be used for event- or tissue-specific regulation of vector gene expression. In addition, multimerization of these sequences (*i.e.*, rev-responsive "CRS/CAR" or tat-responsive "TAR" elements for HIV) may be utilized in order to generate even greater specificity.

In a manner similar to the preceding embodiment, nucleic acids may be generated which carry a gene for phosphorylation, phosphoribosylation, ribosylation, or other metabolism of a purine- or pyrimidine-based drug. Such genes may have no equivalent in mammalian cells, and might come from an organism such as a virus, bacterium, fungus, or protozoan. Representative examples include: *E. coli* guanine phosphoribosyl transferase ("gpt") gene product, which converts thioxanthine into thioxanthine monophosphate (see Besnard et al., *Mol. Cell. Biol.* 7:4139-4141, 1987); alkaline phosphatase, which will convert inactive phosphorylated compounds such as mitomycin phosphate and doxorubicin-phosphate to toxic dephosphorylated compounds; fungal (*e.g.*, *Fusarium oxysporum*) or bacterial cytosine deaminase which will convert 5-fluorocytosine to the toxic compound 5-fluorouracil (Mullen, *PNAS* 89:33, 1992); carboxypeptidase G2 which will cleave the glutamic acid from para-N-bis (2-chloroethyl) aminobenzoyl glutamic acid, thereby creating a toxic benzoic acid mustard; and Penicillin-V amidase, which will convert phenoxyacetabide derivatives of doxorubicin and melphalan to toxic compounds. Conditionally lethal gene products of this type have application to many presently known purine- or pyrimidine-based anticancer drugs, which often require intracellular ribosylation or phosphorylation in order to become effective cytotoxic agents. The conditionally lethal gene product could also metabolize a nontoxic drug, which is not a purine or pyrimidine analogue, to a cytotoxic form (see Searle et al., *Brit. J. Cancer* 53:377-384, 1986).

Additionally, in the instance where the target pathogen is a mammalian virus, the nucleic acid may be constructed to take advantage of the fact that mammalian viruses in general tend to have "immediate early" genes, which are necessary for subsequent transcriptional activation of other viral promoter elements. Gene products of this nature are excellent candidates for intracellular signals (or "identifying agents") of viral infection. Thus, conditionally lethal genes transcribed from transcriptional promoter elements that are responsive to such viral "immediate early" gene products could specifically kill cells

doses, less generalized toxicity, and higher potency against productive infection. Additional nucleoside analogues whose nucleotide triphosphate forms show selectivity for retroviral reverse transcriptase but, as a result of the substrate specificity of cellular nucleoside and nucleotide kinases are not phosphorylated, may also be utilized within the  
5 context of the present invention.

Within a related aspect of the present invention, a nucleic acid directs the expression of a substance that activates another compound with little or no cytotoxicity into a toxic product in the presence of a pathogenic agent, thereby effecting localized therapy to the pathogenic agent. In this case, expression of the gene product from the  
10 nucleic acid is limited to situations wherein an entity associated with the pathogenic agent, such as an intracellular signal identifying the pathogenic state, is present, thereby avoiding destruction of nonpathogenic cells. This cell-type specificity may also be conferred at the level of infection, by targeting the nucleic acid carrying the vector to cells having or being susceptible to the pathogenic condition.

15 Within a related aspect of the present invention, a nucleic acid directs the expression of a gene product(s) that activates a compound with little or no cytotoxicity into a toxic product. Briefly, a wide variety of gene products which either directly or indirectly activate a compound with little or no cytotoxicity into a toxic product may be utilized within the context of the present invention. Representative examples of such gene products  
20 include HSVTK and VZVTK which selectively monophosphorylate certain purine arabinosides and substituted pyrimidine compounds, converting them to cytotoxic or cytostatic metabolites. More specifically, exposure of the drugs gancyclovir, acyclovir, or any of their analogues (*e.g.*, FIAC, DHPG) to HSVTK, phosphorylates the drug into its corresponding active nucleotide triphosphate form.

25 For example, within one embodiment of the invention, the nucleic acid directs the expression of the herpes simplex virus thymidine kinase ("HSVTK") gene downstream, and under the transcriptional control of an HIV promoter (which is known to be transcriptionally silent except when activated by HIV tat protein). Briefly, expression of the tat gene product in human cells infected with HIV and carrying the nucleic acid causes  
30 increased production of HSVTK. The cells (either *in vitro* or *in vivo*) are then exposed to a drug such as gancyclovir, acyclovir or its analogues (FIAC, DHPG). As noted above, these drugs are known to be phosphorylated by HSVTK (but not by cellular thymidine kinase) to their corresponding active nucleotide triphosphate forms. Acyclovir triphosphates inhibit cellular polymerases in general, leading to the specific destruction of  
35 cells expressing HSVTK in transgenic mice (see Borrelli et al., *Proc. Natl. Acad. Sci. USA* 85:7572, 1988). Those cells containing the recombinant vector and expressing HIV tat protein are selectively killed by the presence of a specific dose of these drugs.

antiviral protein (Barbieri et al., *Biochem. J.* 203:55-59, 1982; Irvin et al., *Arch. Biochem. & Biophys.* 200:418-425, 1980; Irvin, *Arch. Biochem. & Biophys.* 169:522-528, 1975), tritin, Shigella toxin (Calderwood et al., *PNAS* 84:4364-4368, 1987; Jackson et al., *Microb. Path.* 2:147-153, 1987), and *Pseudomonas* exotoxin A (Carroll and Collier, *J. Biol. Chem.* 262:8707-8711, 1987).

Within other aspects of the invention, the nucleic acid carries a gene specifying a product which is not in itself toxic, but when processed or modified by a protein, such as a protease specific to a viral or other pathogen, is converted into a toxic form. For example, the recombinant retrovirus nucleic acid could carry a gene encoding a  
10 proprotein chain, which becomes toxic upon processing by the HIV protease. More specifically, a synthetic inactive proprotein form of the toxic ricin or diphtheria A chains could be cleaved to the active form by arranging for the HIV virally encoded protease to recognize and cleave off an appropriate "pro" element.

Within yet another aspect of the invention, the nucleic acid directs the  
15 expression of a substance capable of activating an otherwise inactive precursor into an active inhibitor of a pathogenic agent, or a conditional toxic palliative, which are palliatives that are toxic for the cell expressing the pathogenic condition. As will be evident to one of skill in the art given the disclosure provided herein, a wide variety of inactive precursors may be converted into active inhibitors of a pathogenic agent. For example, antiviral  
20 nucleoside analogues such as AZT or ddI are metabolized by cellular mechanisms to the nucleotide triphosphate form in order to specifically inhibit retroviral reverse transcriptase, and thus viral replication (Furmam et al., *Proc. Natl. Acad. Sci. USA* 83:8333-8337, 1986). Recombinant viral vectors which direct the expression of a gene product (e.g., a protein) such as Herpes Simplex Virus Thymidine Kinase (HSVTK) or Varicella Zoster Virus  
25 Thymidine Kinase (VZVTK) which assists in metabolizing antiviral nucleoside analogues to their active form are therefore useful in activating nucleoside analogue precursors (e.g., AZT or ddC) into their active form. AZT or ddI therapy will thereby be more effective, allowing lower doses, less generalized toxicity, and higher potency against productive infection. Additional nucleoside analogues whose nucleotide triphosphate forms show  
30 selectivity for retroviral reverse transcriptase but, as a result of the substrate specificity of cellular nucleoside and nucleotide kinases are not phosphorylated, will be made more efficacious.

Within one embodiment of the invention, the HSVTK gene may be expressed under the control of a constitutive macrophage or T-cell-specific promoter, and  
35 introduced into macrophage or T-cells. Constitutive expression of HSVTK results in more effective metabolism of nucleotide analogues such as AZT or ddI to their biologically active nucleotide triphosphate form, and thereby provides greater efficacy, delivery of lower

inappropriately expresses a retroviral vector (e.g., in the wrong cell type), or that has become tumorigenic due to inappropriate insertion into a host cell's genome.

A wide variety of nucleic acid molecules may be carried by the complex or gene delivery vehicle of the present invention. Examples of such nucleic acid molecules  
5 include genes and other nucleic acid molecules that encode a substance, as well as biologically active nucleic acid molecules such as inactivating sequences that incorporate into a specified intracellular nucleic acid molecule and inactivate that molecule. A nucleic acid molecule is biologically active when the molecule itself provides the desired benefit without requiring the expression of a substance. For example, the biologically active  
10 nucleic acid molecule may be an inactivating sequence that incorporates into a specified intracellular nucleic acid molecule and inactivates that molecule, or the molecule may be a tRNA, rRNA or mRNA that has a configuration that provides a binding capability.

Substances include proteins (e.g., antibodies including single chain molecules), immunostimulatory molecules (such as antigens) immunosuppressive  
15 molecules, blocking agents, and palliatives (such as toxins, antisense ribonucleic acids, ribozymes, enzymes, and other material capable of inhibiting a function of a pathogenic agent). Within the present invention, "capable of inhibiting a function" means that the palliative either directly inhibits the function or indirectly does so, for example, by converting an agent present in the cells from one which would not normally inhibit a  
20 function of the pathogenic agent to one which does. Examples of such functions for viral diseases include adsorption, replication, gene expression, assembly, and exit of the virus from infected cells. Examples of such functions for cancerous diseases include cell replication, susceptibility to external signals (e.g., contact inhibition), and lack of production of anti-oncogene proteins.

25 Within one embodiment of the present invention, a method is provided for administration of various nucleic acids, such as eukaryotic viral cDNA expression vectors, which direct the expression of a palliative as a DNA molecule. Within another embodiment of the present invention, a method is provided for administration of various nucleic acids which direct the expression of a palliative as an RNA molecule.

30 Representative examples of palliatives that act directly to inhibit the growth of cells include toxins such as ricin (Lamb et al., *Eur. J. Biochem.* 148:265-270, 1985), abrin (Wood et al., *Eur. J. Biochem.* 198:723-732, 1991; Evensen et al., *J. of Biol. Chem.* 266:6848-6852, 1991; Collins et al., *J. of Biol. Chem.* 265:8665-8669, 1990; Chen et al., *Fed. of Eur. Biochem Soc.* 309:115-118, 1992), diphtheria toxin (Tweten et al., *J. Biol.*  
35 *Chem.* 260:10392-10394, 1985), cholera toxin (Mekalanos et al., *Nature* 306:551-557, 1983; Sanchez & Holmgren, *PNAS* 86:481-485, 1989), gelonin (Stirpe et al., *J. Biol. Chem.* 255:6947-6953, 1980), pokeweed toxin (Irvin, *Pharmac. Ther.* 21:371-387, 1983),



growth hormone, cell surface receptors, low density lipoproteins, transferrin, erythropoietin, insulin and fibrinolytic enzymes. Other targeting elements include immune accessory molecules, which include, for example, the interleukins IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-11, IL-12, IL-13, IL-14, IL-15,  $\alpha$ -interferon,  $\beta$ -interferon,  $\gamma$ -interferon, GM-CSF, G-CSF, G-CSF and M-CSF. With the aforementioned ligands, a desired cell type which expresses the binding partner is targeted due to the choice of the appropriate ligand. Alternatively, one member of a wide variety of high affinity binding pairs may also be used as a ligand. These include, for example, biotin/avidin, cytochrome c/papain, valphosphonate/carboxypeptidase A, 4CABP/RuBisCo, and tobacco hornworm diuretic hormone/tobacco hornworm diuretic hormone receptor, as well as antigen/antibody binding pairs. When using these high affinity binding pairs one member of the pair is incorporated into the nucleic acid/condensing agent complex and the other member attached to the cell of interest. The ligands may be coupled to the condensing agent by methods described in U.S. Patent Nos. 5,166,320 and 5,354,844 (incorporated herein by reference).

Additionally, components which disrupt endosomes may be included in the complex. For example, the capsid proteins of adenoviruses cause acidification of an endosome and ultimate disruption, thereby releasing the introduced nucleic acid (Defer et al., *J. Virol.* 64:3661, 1990; Seth et al., *Mol. Cell. Biol.* 4:1528, 1984; Fitzgerald et al., *Cell* 32:607, 1983). Through this mechanism, adenovirus can greatly increase the efficiency of gene transfer (Curiel et al., *Proc. Natl. Acad. Sci USA* 88:8850, 1991). Other endosomolytic agents may alternatively be incorporated into the complex to achieve an endosome breakdown. Other agents, such as lysomotropic agents (e.g., chloroquine), may be included to decrease DNA degradation that occurs in lysosomes.

25

#### NUCLEIC ACIDS

A nucleic acid molecule administered to an animal in accordance with the present invention does not naturally occur in the complex or gene delivery vehicle that carries it, and is neither inert nor generally harmful to the animal, but rather provides some desirable benefit, typically an ability to fight a disease or other pathogenic agent. As used herein, "pathogenic agent" refers to a cell that is responsible for a disease state. Representative examples of pathogenic agents include tumor cells, autoreactive immune cells, hormone secreting cells, cells which lack a function that they would normally have, cells that have an additional inappropriate gene expression which does not normally occur in that cell type, and cells infected with bacteria, viruses, or other intracellular parasites. In addition, as used herein, "pathogenic agent" may also refer to a cell that over-expresses or

30  
35

with the retroviral vector. Non-specific binding sites are blocked with bovine serum albumin (BSA), and wells are exposed to serial dilutions of either individual or pooled mouse sera. The wells are washed thoroughly and incubated with commercially available goat anti-mouse immunoglobulins conjugated to horseradish peroxidase (HRP) or alkaline phosphatase (AP). The color is developed with the ortho-phenyldiamine in hydrogen peroxide solution.

A variety of other immunoassay methods can also be used. (See eg. Enzyme Immunoassay, E.T. Maggio, editor, CRC Press, Boca Raton, Florida (1980.)) For instance, a capture ELISA assay can be set up in the following manner. Microtiter well plates can be coated with serial dilutions of mouse sera, and non specific binding sites can be blocked with a solution of BSA. A preparation of retroviral vector is then added to the wells, which are washed thoroughly and exposed to monoclonal anti-vector antibodies. The wells are then treated with commercially available goat anti-mouse antibodies conjugated with either HRP or AP, and the assay is performed as described above.

Synthetic polymers are known to exhibit varying levels of immunogenicity depending upon the polymer itself, the type of conjugation to the protein, or the nature of the protein carrier. Dextrans of high molecular weight (>95,000) are known to be immunogenic, especially when bound to protein carriers (Kabat, E. A. and Berg, D.J. *Immunology* 70:514, 1953; Richter, W. and Kagedal, L. *Int. Arch. Allergy* 42:885, 1972). Therefore, the immune response against the dextran portion of dextran-modified vectors can also be evaluated in sera of animals with a modification of the immunization procedures and immunoassays described above. For example, microtiter well plates can be coated with unmodified vector, dextran-modified vector, BSA, and dextran-modified BSA. Following a wash, non-specific binding sites are blocked with a BSA solution, and the wells are exposed to serum samples, either individually or pooled. The wells are again washed thoroughly. A preparation of rabbit anti-BSA IgG can be used as a control and the immunogenicity of the synthetic polymer can be calculated.

#### 30 ADDITIONAL COMPONENTS IN NUCLEIC ACID/CONDENSING AGENT COMPLEXES

Within various aspects of the invention, a wide variety of ligands are provided. Ligands which bind to a cell surface protein, may be included in the complex to facilitate targeting of the condensed nucleic acid complex to a particular cell type. Such ligands include, for example, transferrin, asialoglycoprotein, antibody and antibody fragments, bombesin, gastrin-release peptide, cell adhesion peptides, substance P, neuromedin-B, neuromedin-C, metenkephalin, EGF, alpha- and beta-TGF, neurotensin, melanocyte stimulating hormone, follicle stimulating hormone, luteinizing hormone, human

Because measurements of total antibody by ELISA do not measure the level of antibody that is "neutralizing" (i.e., prevents the complex from achieving gene transfer) selection of condensing agents that are immunogenic may be justified if antibodies do not inhibit gene transfer. Interference with *in vitro* gene transfer efficiency is determined after  
5 treatment with heat-inactivated sera from multiply-injected animals and after treatment with heat-inactivated FBS. Efficiencies are determined for DNA complexed with the candidate condensing agent as well as with polylysine. The relative efficiency is calculated as a percentage for each condensing agent in the presence of immune sera compared to FBS. A nucleic acid/condensing agent complex will meet the criteria for low immunogenicity if its  
10 relative efficiency is at least three-fold, and preferably ten-fold, higher than the relative efficiency for the nucleic acid/polylysine complex.

If the condensing agent does not have low immunogenicity by ELISA after *in vivo* administration or elicits neutralizing antibodies, it may still be considered useful within the context of this invention, if it is not inactivated by a serum component. Such an  
15 agent may be used when a single dose of gene therapy is to be administered. Inactivation by a serum component may be mediated by one of the proteins of the complement system. Briefly, in this assay, nucleic acid, conveniently a plasmid expressing  $\beta$ -galactosidase, such as pSV- $\beta$  Galactosidase (obtainable from Promega, Cat# E1081), is complexed with the candidate nucleic acid condensing agent. The complex is then treated with heat-inactivated  
20 fetal bovine sera (as a control), human sera, or heat-inactivated human sera. Heat-inactivated sera are prepared by heating to 56°C for 30 minutes. These mixtures are then incubated at 37°C for 30 minutes. Transfection efficiency is determined by a standard blue colony forming unit (BCFU) assay (*Current Protocols in Molecular Biology, supra*). Reduced sensitivity to inactivation and thus, low immunogenicity, is defined as an increase  
25 of two-fold or better survival relative to polylysine-condensed DNA. Survival is expressed as BCFU/ml after treatment with heat-inactivated serum relative to treatment with FBS.

The immunogenicity of polymer-modified vectors can be evaluated in a variety of animal models by standard immunology techniques. (See Harlow and Lane, Antibodies: A Laboratory Manual, Cold Spring Harbor Publications (1988.)) Typically, an  
30 immunization and bleeding schedule is first determined. Prior to the initiation of the experiment, animals can be pre-bled and their preexisting anti-vector titres determined, for instance, with an ELISA assay.

Animals can be injected with the modified vectors of the invention and the immune responses can be determined by immunological methods. For example, the  
35 immunogenicity of modified retroviral vectors in mice can be determined by measuring the extent of the anti-vector immune response in mouse sera with an ELISA assay (Engvall, E. *Meth. Enzymol* 70:419, 1980). In this particular assay, microtiter well plates are coated

complex in a standard ELISA. Wells are coated with a polyclonal antibody specific for the unconjugated condensing agent/nucleic acid complex (*i.e.*, antibody obtained from sera of rabbits multiply injected with polylysine/nucleic acid with or without an adjuvant). Injections are continued until an adequate antibody titer is obtained in serum. Alternatively, but less preferably, a monoclonal antibody may be used. Conjugations which decrease the binding by at least three fold, or ten-fold preferably, relative to the binding to unmodified condensing agent/nucleic acid complex will be chosen as promising candidates for *in vivo* testing.

An *in vivo* assay for immunogenicity is performed by introducing the conjugated condensing agent or novel condensing agent *in vivo* and determining an antibody immune response. In this assay, mice, rabbits, or preferably macaques or other primates, are repeatedly injected with nucleic acids condensed with one of the condensing agents described above. As an illustration, a plasmid, such as pGL2, is condensed with PEG-conjugated polylysine and injected *i.m.*, *i.v.*, *i.p.*, or *s.c.* into a group of three or more young adult mice. A total of three injections are given with an interval between injections ranging from two weeks to two months. The dose per injection in each group may be based on projection of the approximate human dose equivalent for the specific gene and gene transfer application being considered and may be bracketed 3-10 fold higher and lower in parallel groups. Alternatively, the dose may be chosen arbitrarily at 100 mg/kg and may be bracketed 3-10 fold higher and lower for alternative groups, or the dose may be established by first determining a dose of unconjugated polycation that gives a measurable antibody response. Serum is isolated from blood samples collected at 1, 2, 3, 4, and 8 weeks after the final injection. Antibody titers against the condensing agent are measured by ELISA and compared to antibody titers against unconjugated polylysine-condensed complexes obtained from mice injected with these complexes. Lowered immunogenicity is defined as a reduction of induced antibody titer of at least three-fold, and preferably ten-fold or greater relative to the polylysine/nucleic acid complex injected animal, at matched DNA dosage. Satisfying this criteria at any of the dose/interval combinations tested in an animal system is sufficient to show low immunogenicity within the context of this invention.

In the case of the novel condensing agents, immunogenicity studies are preferably done in animals closely related to, and most preferably identical, to the species for which the gene transfer complex is intended. That is, gene transfer complexes using novel condensing agents which are intended for human use are tested for *in vivo* immunogenicity preferably in primates and most preferably in humans. In such cases, the control standard against which immunogenicity comparisons are based remains the nucleic acid/unconjugated-polylysine complex.

luciferase are determined using an assay kit (Promega Corp. Part No. E1500) according to manufacturer's instructions.

Gene transfer efficiency of the plasmid condensed with the candidate condensing agent is compared to the efficiency obtained with the plasmid condensed with polylysine having an average length of 270 amino acids. Agents that mediate gene transfer with an efficiency of at least 10%, and preferably 50% or more, relative to polylysine are chosen for further analysis.

### 3. Immunogenicity

Following the identification of agents which condense DNA and determination of the efficiency of gene transfer *in vitro*, these agents are assayed for immunogenicity. *In vivo* tests may be performed and are generally preferred. In assessing whether an agent elicits an immune response, the most important responses to evaluate are an antibody response and other humoral factors which may interfere with *in vivo* function, either initially or after repeated doses to a subject.

Nucleic acid condensed with the modified condensing agent or novel condensing agent is introduced *in vivo* and an antibody immune response determined. If the total antibody response as measured by ELISA is sufficiently low, the candidate agent is selected. If this antibody response is not sufficiently low, sera is tested in a neutralization assay. If the antibodies are neutralizing, the candidate is discarded. If the antibodies are not neutralizing, the candidate agent is selected. Selected candidates may be tested further for inactivation by a serum component.

The antibody immune response is tested differently for condensing agents that are conjugated polycations and for condensing agents that are basic amino acid regions. For the first type of condensing agents, modifications of a particular condensing agent/nucleic acid complex relative to the unmodified complex may be initially and optionally tested by an *in vitro* test, such as an ELISA as described below, in order to screen for the most promising modifications of condensing agents to be tested by *in vivo* assays. For all types of agents, *in vivo* determinations of immunogenicity are performed and preferred. In assessing the ability of an agent to elicit an immune response, an antibody response is a critical parameter to evaluate as described in Example 5. Additionally, other parameters to be evaluated include assessment of other humoral factors, such as complement. An example of an assay to address the presence of these factors is set forth in Example 6. These factors may interfere with *in vivo* function either initially or after repeated administrations of a particular condensing agent/nucleic acid complex.

In an *in vitro* assay, antibody reactivity to a specific conjugated nucleic acid condensing agent/nucleic acid complex is compared to the unmodified condensing agent

will not intercalate as much ethidium bromide or other intercalating dye and has reduced mobility in agarose gel electrophoresis.

As an illustrative example, an intercalating dye assay using ethidium bromide is described. In this assay, test nucleic acids, conveniently plasmid DNA, are mixed with  
5 candidate condensing agents from a 1:1 to a 1:50 w/w ratio of plasmid to condensing agent. Following incubation, ethidium bromide is added to the reaction to a final concentration of 1  $\mu\text{g/ml}$ . If a nucleic acid such as RNA is used as the test nucleic acid, acridine orange may be used as the intercalating dye. The reaction mixtures are transferred into UV transparent plastic tubes spotted onto 1% agarose gel, or placed upon UV  
10 transparent plastic film and illuminated with 260 nm light. The emission from the DNA-ethidium bromide complex is recorded on film by a camera equipped with an appropriate UV filter. The ability of the candidate condensing agent to condense DNA is inversely proportional to the intensity of the fluorescence in each reaction mixture. A condensing agent which either reduces fluorescence intensity by at least 10-fold relative to  
15 uncondensed plasmid or reduces fluorescence intensity to 50% of the reduction caused by polylysine condensation is selected for further analysis.

The more precise test, and the one performed for each candidate condensing agent, is a band shift assay. Briefly, this assay is performed by incubating nucleic acids, either labeled or unlabeled, with various concentrations of candidate condensing agents.  
20 Test nucleic acids, conveniently plasmid DNA, and condensing agent are mixed at 1:1 to 1:50 w/w ratios. Following incubation, each sample is loaded on a 1% agarose gel and electrophoresed. The gel is then either stained with ethidium bromide or dried and autoradiographed. DNA condensation is determined by the inability to enter the gel compared to a non-condensed standard. Sufficient condensation is achieved when at least  
25 90% of the DNA fails to enter the gel to any significant degree.

## 2. Efficiency of Gene Transfer

A variety of *in vitro* methods may be utilized in order to assess the efficacy of gene transfer with these nucleic acid condensing agents. For example, an *in vitro* test  
30 may be performed using a plasmid construct containing a reporter gene such as  $\beta$ -gal, luciferase, CAT, or human growth hormone. An appropriate recipient cell line is chosen for the assay. This cell line is preferably a cell line with properties similar to the cells to which the gene will be targeted in patients. However, the recipient cells should not express significant levels of the same gene product that is contained on the test plasmid. Of the  
35 various reporter genes, luciferase is used herein as an example. Briefly, the reporter gene construct is mixed with condensing agents. Following an incubation period, the mixtures are added to the recipient cells. Cells are harvested 1 day after transfer. The levels of

If single units of basic amino acid regions derived from identified basic proteins, such as chromosomal proteins, transcription factors, DNA virus coat proteins, or other proteins are less than 10% as efficient as polylysine in gene transfer assays, multiple units may be synthesized and tested for sufficient gene transfer efficiency.

5 One embodiment of this invention provides a condensing agent that contains multiple units of basic domains from native protein. Briefly, a region from a basic protein is identified as described above. DNA molecules encoding the basic region may be ligated together in a head-to-tail or head-to-head fashion following the addition of adapters containing appropriate restriction sites. From 1-10 units are composed in an array,  
10 preferably 1-5 units, most preferably 1-2. Alternatively, restriction sites may be added during amplification of the region by incorporating a restriction site sequence into the primers used in amplification. Following ligation of the units, molecules containing the desired number of repeat units may be isolated following gel electrophoresis. These units are then inserted into an appropriate expression vector (which may be commercially  
15 obtained), propagated, and protein isolated by conventional techniques. Alternatively, multiple units may be generated chemically by synthesizing single units of basic amino acid regions and chemically or enzymatically coupling them together.

#### ANALYSIS OF CONDENSING AGENTS

20 Once condensing agents have been prepared as described above, they are tested for several different parameters. First, the candidate condensing agent must be able to condense nucleic acids. Second, nucleic acids are condensed by the agent and tested in an *in vitro* gene transfer assay. Third, the agent is tested as part of a condensed nucleic acid-complex immunogenicity assay, both for induction of antibodies and inactivation by a  
25 serum component. Only condensing agents satisfying the criteria of the first, second, and either part of the third assay are selected as condensing agents with low immunogenicity. Thus, the nucleic acid condensing agents identified as described herein possess low or negligible immunogenicity, while retaining substantial efficiency of gene transfer activity.

30

#### 1. Condensation

Agents which have been modified or newly identified are assayed for their ability to condense nucleic acid utilizing at least two different assays. An intercalating dye assay may be used as an optional, preliminary screen. All candidate condensing agents are assayed by a DNA band shift assay. Both assays are based upon changes in the physical  
35 properties of DNA when it is condensed. Condensation "collapses" DNA into macromolecular structures, commonly into a toroid form. In this condensed state, DNA

enzyme digestion, before cloning and expression in bacteria, yeast, or other eukaryotic cells.

Proteins satisfying the criteria discussed above for basicity may be found by interrogating a database of protein sequences, such as PIR (Protein Identification Resource) or Swiss Prot. Database sequences are first scanned for proteins with a predicted isoelectric point of at least 8. Among those, the proteins are scanned for stretches of at least 50 amino acids containing at least 40% basic (generally, lysine plus arginine) residues, not greater than 5% acidic residues, and a predicted isoelectric point of at least 9. Alternatively, the database may be scanned in 50 amino acid blocks for stretches with the desired characteristics. A preferred length for a basic amino acid region is at least 90 amino acids and a particularly preferred length is at least 200 amino acids. Analysis indicates that proteins such as human serum albumin, DNA binding proteins, non-histone chromosomal proteins, proteins with nucleic acid binding capacity, or others, have regions exhibiting the requisite characteristics. Preferred regions are from the same species as the ultimate host for gene delivery.

Once such proteins are identified, the whole protein or basic domain, either in its native form or conjugated as described above, is tested for its ability to condense DNA, *in vitro* gene transfer efficiency, and for immunogenicity as complexed with nucleic acid.

A basic amino acid region is tested for *in vitro* gene transfer efficiency at a ratio that causes condensation of DNA as assayed in a band shift assay. If a single unit of basic amino acid region condensed DNA has at least 10% the efficiency of polylysine condensed DNA, it is tested in an immunogenicity assay. Preferably, *in vitro* gene transfer efficiency is at least 50% of the efficiency of polylysine.

Also within the scope of this invention, coat proteins from DNA viruses may serve as nucleic acid condensing agents. Coat proteins bind DNA and assist in the packaging of viral genomes into viral capsids. One coat protein, from  $\phi$ X174, is known to condense DNA. Other coat proteins may be prepared chemically or biologically and tested for their ability to condense DNA, transfer genes *in vitro*, and for immunogenicity.

Transcriptional factors also contain domains that bind DNA and therefore may be useful as nucleic acid condensing agents. Briefly, transcriptional factors such as C/EBP, *c-jun*, *c-fos*, AP-1, AP-2, AP-3, CPF, Prr-1, Sp-1, Oct-1, Oct-2, CREP, and TFIID contain basic domains that bind DNA sequences. As discussed above, the basic domain may be prepared by PCR amplification, isolated by restriction enzyme digestion, or chemically synthesized. Single units are tested for nucleic acid condensation, *in vitro* gene transfer efficiency, and immunogenicity.



As will be evident to one of ordinary skill in the art given the disclosure provided herein, when utilizing viral gene delivery vehicles, the efficiency of packaging and hence, viral titer, is to some degree dependent upon the size of the sequence to be packaged. Thus, in order to increase the efficiency of packaging and the production  
5 particle virus, additional non-coding sequences may be added to the gene delivery vehicle. Moreover, within certain embodiments of the invention it may be desired to increase or decrease viral titer. This increase or decrease may be accomplished by increasing or decreasing the size of the heterologous sequence, and hence the efficiency of packaging.

Within one embodiment, gene delivery vehicles may be constructed to  
10 include a promoter such as SV40 (*see*, Kriegler et al., *Cell* 38:483, 1984), cytomegalovirus ("CMV") (*see*, Boshart et al., *Cell* 41:521-530, 1991), or an internal ribosomal binding site ("IRBS"). Briefly, with respect to IRBS, the upstream untranslated region of the immunoglobulin heavy chain binding protein has been shown to support the internal engagement of a bicistronic message (*see*, Jacejak and Sarnow, *Nature* 353:90-94, 1991).  
15 This sequence is small (300 bp), and may readily be incorporated into a retroviral vector or other gene delivery vehicle in order to express multiple genes from a multi-cistronic message whose cistrons begin with this sequence.

## 2. Identification of novel condensing agents

20 This invention also provides new condensing agents, with low or negligible immunogenicity, which are useful as condensing agents for nucleic acid. In one embodiment, the condensing agent is a basic amino acid region of at least 50 amino acids that has a predicted isoelectric point of at least 9, contains at least 40% basic amino acids, but not greater than 5% acidic amino acids, and preferably no acidic amino acids.  
25 Computer programs, such as Geneworks, version 2.4 (Intelligenetics), may be used as an aid in the calculation of isoelectric point.

A basic amino acid region is identified by its physical characteristics, predicted isoelectric point and percentage of basic amino acids. These regions may be derived from native proteins or contain a synthesized sequence not corresponding to a  
30 native protein. These regions must be at least 50 amino acids long, preferably longer than 90 residues, and particularly preferred longer than 200 amino acids. The total percentage of basic residues (generally, arginine plus lysine) for a native protein must be at least 40% and preferably greater than 50%. Histones and protamines are examples of proteins which have regions that satisfy these criteria. Once a basic region is identified, either the region  
35 may be synthesized based on the amino acid sequence, or the corresponding region of the gene may be isolated by conventional techniques, such as PCR amplification or restriction

packaging sequence resides within the nonstructural coding region, therefore only the genomic 49S RNA is packaged into virions.

Several different Sindbis vector systems may be constructed and utilized within the present invention. Representative examples of such systems include those  
5 described within U.S. Patent Nos. 5,091,309 and 5,217,879.

Particularly preferred Sindbis vectors for use within the present invention include those which are described within U.S. Serial No. 08/198,450. Briefly, within one embodiment, Sindbis vector constructs are provided comprising a 5' sequence which is capable of initiating transcription of a Sindbis virus, a nucleotide sequence encoding Sindbis  
10 non-structural proteins, a viral junction region which has been inactivated such that viral transcription of the subgenomic fragment is prevented, and a Sindbis RNA polymerase recognition sequence. Within other embodiments, the viral junction region has been modified such that viral transcription of the subgenomic fragment is reduced. Within another embodiment, Sindbis vector constructs are provided comprising a 5' sequence  
15 which is capable of initiating transcription of a Sindbis virus, a nucleotide sequence encoding Sindbis non-structural proteins, a first viral junction region which has been inactivated such that viral transcription of the subgenomic fragment is prevented, a second viral junction region which has been modified such that viral transcription of the subgenomic fragment is reduced, and a Sindbis RNA polymerase recognition sequence.  
20 Within yet another embodiment, Sindbis cDNA vector constructs are provided comprising the above-described vector constructs, in addition to a 5' promoter which is capable of initiating the synthesis of viral RNA from cDNA, and a 3' sequence which controls transcription termination.

In still further embodiments, the vector constructs described above contain  
25 no Sindbis structural proteins in the vector constructs the selected heterologous sequence may be located downstream from the viral junction region; in the vector constructs described above having a second viral junction, the selected heterologous sequence may be located downstream from the second viral junction region, where the heterologous sequence is located downstream, the vector construct may comprise a polylinker located  
30 between the viral junction region and said heterologous sequence, and preferably the polylinker does not contain a wild-type Sindbis virus restriction endonuclease recognition sequence.

When Sindbis gene delivery vehicles are utilized, the modified cells are fully viable and healthy, and no other viral antigens (which may well be immunodominant) are  
35 expressed. The above-described Sindbis vector constructs, as well as numerous similar vector constructs, may be readily prepared essentially as described in U.S. Serial No. 08/198,450, which is incorporated herein by reference in its entirety.

such retrovector constructs are utilized, it is preferable to utilize packaging cell lines for the production of recombinant viral particles wherein the 5' terminal end of the *gag/pol* gene in a *gag/pol* expression cassette has been modified to contain codons which are degenerate for *gag*.

5                Within other aspects of the present invention, retrovector constructs are provided comprising a 5' LTR, a tRNA binding site, a packaging signal, an origin of second strand DNA synthesis and a 3' LTR, wherein the retrovector construct does not contain a retroviral nucleic acid sequence upstream of the 5' LTR. As utilized within the context of the present invention, the phrase "does not contain a retroviral nucleic acid sequence  
10 upstream of the 5' LTR" should be understood to mean that the retrovector does not contain at least 20, preferably at least 15, more preferably at least 10, and most preferably at least 8 consecutive nucleotides which are found in a retrovirus, and more specifically, in a retrovirus which is homologous to the retrovector construct. Within a preferred embodiment, the retrovector constructs do not contain a *env* coding sequence upstream of  
15 the 5' LTR.

              Within a further aspect of the present invention, retrovector constructs are provided, comprising a 5' LTR, a tRNA binding site, a packaging signal, an origin of second strand DNA synthesis and a 3' LTR, wherein the retrovector construct does not contain a retroviral packaging signal sequence downstream of the 3' LTR. As utilized  
20 herein, the term "packaging signal sequence" should be understood to mean a sequence sufficient to allow packaging of the RNA genome.

              Within another preferred embodiment, the gene delivery vehicle is a Sindbis viral vector. Briefly, the Sindbis virus is the prototype member of the alphavirus genus of the togavirus family. The unsegmented genomic RNA (49S RNA) of Sindbis virus is  
25 approximately 11,703 nucleotides in length, contains a 5' cap and a 3' polyadenylated tail, and displays positive polarity. Infectious enveloped Sindbis virus is produced by assembly of the viral nucleocapsid proteins onto the viral genomic RNA in the cytoplasm and budding through the cell membrane embedded with viral encoded glycoproteins. Entry of virus into cells is by endocytosis through clathrin coated pits, fusion of the viral membrane  
30 with the endosome, release of the nucleocapsid, and uncoating of the viral genome. During viral replication the genomic 49S RNA serves as template for synthesis of the complementary negative strand. This negative strand in turn serves as template for genomic RNA and an internally initiated 26S subgenomic RNA. The Sindbis viral nonstructural proteins are translated from the genomic RNA while structural proteins are  
35 translated from the subgenomic 26S RNA. All viral genes are expressed as a polyprotein and processed into individual proteins by post-translational proteolytic cleavage. The

Watson-Crick base pairing, and is carried with the retrovirus genome into a viral particle. The tRNA is then utilized as a primer for DNA synthesis by reverse transcriptase. The tRNA binding site may be readily identified based upon its location just downstream from the 5' LTR. Similarly, the origin of second strand DNA synthesis is, as its name implies, important for the second strand DNA synthesis of a retrovirus. This region, which is also referred to as the poly-purine tract, is located just upstream of the 3' LTR.

In addition to a 5' and 3' LTR, tRNA binding site, and origin of second strand DNA synthesis, certain preferred retrovector constructs which are provided herein also comprise a packaging signal, as well as one or more heterologous sequences, each of which is discussed in more detail below.

Within one aspect of the invention, retrovector constructs are provided which lack both *gag/pol* and *env* coding sequences. As utilized herein, the phrase "lacks *gag/pol* or *env* coding sequences" should be understood to mean that the retrovector does not contain at least 20, preferably at least 15, more preferably at least 10, and most preferably at least 8 consecutive nucleotides which are found in *gag/pol* or *env* genes, and in particular, within *gag/pol* or *env* expression cassettes that are used to construct packaging cell lines for the retrovector construct.

As an illustration, within one embodiment of the invention construction of retrovector constructs which lack *gag/pol* or *env* sequences may be accomplished by preparing vector constructs which lack an extended packaging signal. As utilized herein, the phrase "extended packaging signal" refers to a sequence of nucleotides beyond the minimum core sequence which is required for packaging, that allows increased viral titer due to enhanced packaging. As an example, for the Murine Leukemia Virus MoMLV, the minimum core packaging signal is encoded by the sequence beginning from the end of the 5' LTR up through the *Pst* I site. The extended packaging signal of MoMLV includes the sequence beyond nucleotide 567 up through the start of the *gag/pol* gene (nucleotide 621), and beyond nucleotide 1560. Thus, within this embodiment, retrovector constructs which lack extended packaging signal may be constructed from the MoMLV by deleting or truncating the packaging signal prior to nucleotide 567.

Within other embodiments of the invention, retrovector constructs are provided wherein the packaging signal that extends into, or overlaps with, retroviral *gag/pol* sequence is deleted or truncated. For example, in the representative case of MoMLV, the packaging signal is deleted or truncated prior to the start of the *gag/pol* gene. Within preferred embodiments of the invention, the packaging signal is terminated at nucleotide 570, 575, 580, 585, 590, 595, 600, 610, 615, or 617.

Within other aspects of the invention, retrovector constructs are provided which include a packaging signal that extends beyond the start of the *gag/pol* gene. When

Particularly preferred retroviruses for the preparation or construction of retroviral gene delivery vehicles of the present invention include retroviruses such as Avian Leukosis Virus, Bovine Leukemia Virus, Murine Leukemia Virus, Mink Cell Focus-Inducing Virus, Murine Sarcoma Virus, Reticuloendotheliosis Virus, and Rous Sarcoma Virus. Particularly preferred Murine Leukemia Viruses include 4070A and 1504A (Hartley and Rowe, *J. Virol.* 19:19-25, 1976), Abelson (ATCC No. VR-999), Friend (ATCC No. VR-245), Graffi, Gross (ATCC No. VR-590), Kirsten Sarcoma Virus, Harvey Sarcoma Virus, Rauscher (ATCC No. VR-998), and Moloney Murine Leukemia Virus (ATCC No. VR-190). Particularly preferred Rous Sarcoma Viruses include Bratislava, Bryan high titer (*e.g.*, ATCC Nos. VR-334, VR-657, VR-726, VR-659, and VR-728), Bryan standard, Carr-Zilber, Engelbreth-Holm, Harris, Prague (*e.g.*, ATCC Nos. VR-772 and 45033), and Schmidt-Ruppin (*e.g.* ATCC Nos. VR-724, VR-725, VR-354).

Any of the above retroviruses may be readily utilized in order to assemble or construct retroviral gene delivery vehicles given the disclosure provided herein, and standard recombinant techniques (*e.g.*, Sambrook et al, *Molecular Cloning: A Laboratory Manual*, 2d ed., Cold Spring Harbor Laboratory Press, 1989; Kunkle, *PNAS* 82:488, 1985). In addition, within certain embodiments of the invention, portions of the retroviral gene delivery vehicles may be derived from different retroviruses. For example, within one embodiment of the invention, retrovector LTRs may be derived from a Murine Sarcoma Virus, a tRNA binding site from a Rous Sarcoma Virus, a packaging signal from a Murine Leukemia Virus, and an origin of second strand synthesis from an Avian Leukosis Virus.

Within one aspect of the present invention, retrovector constructs are provided comprising a 5' LTR, a tRNA binding site, a packaging signal, one or more heterologous sequences, an origin of second strand DNA synthesis and a 3' LTR, wherein the vector construct lacks *gag/pol* or *env* coding sequences. Briefly, LTRs are subdivided into three elements, designated U5, R, and U3. These elements contain a variety of signals which are responsible for the biological activity of a retrovirus, including for example, promoter and enhancer elements which are located within U3. LTRs may be readily identified in the provirus due to their precise duplication at either end of the genome. As utilized herein, a 5' LTR should be understood to include a 5' promoter element and sufficient LTR sequence to allow reverse transcription and integration of the DNA form of the vector. The 3' LTR should be understood to include a polyadenylation signal sequence, and sufficient LTR sequence to allow reverse transcription and integration of the DNA form of the vector.

The tRNA binding site and origin of second strand DNA synthesis are also important for a retrovirus to be biologically active, and may be readily identified by one of skill in the art. For example, retroviral tRNA binds to a tRNA binding site by

consists of regions of the genome that is finally packaged into the viral particle. These regions include the packaging signal, long terminal repeats (LTR) with promoters and polyadenylation signal sequences, and two start sites for DNA replication. The internal or "trans-acting" part of the cloned provirus is replaced by the gene of interest to create a  
5 "vector construct". When the vector construct is placed into a cell where viral packaging proteins are present (*see*, U.S.S.N. 07/800,921), the transcribed RNA will be packaged as a viral particle which, in turn, will bud off from the cell. These particles are used to transduce tissue cells, allowing the vector construct to integrate into the cell genome. Although the vector construct expresses its gene product, the virus carrying it is replication  
10 defective because the trans-acting portion of the viral genome is absent. Various assays may be utilized in order to detect the presence of any replication competent infectious retrovirus. One preferred assay is the extended S<sup>+</sup>L<sup>-</sup> assay.

Numerous retroviral gene delivery vehicles may be utilized within the context of the present invention, including for example those disclosed within EP 415,731;  
15 WO 90/07936; WO 91/0285; WO 94/03622; WO 93/25698; WO 93/25234; U.S. Patent No. 5,219,740; WO 93/11230; WO 93/10218; Vile and Hart, *Cancer Res.* 53:3860-3864, 1993; Vile and Hart, *Cancer Res.* 53:962-967, 1993; Ram et al., *Cancer Res.* 53:83-88, 1993; Takamiya et al., *J. Neurosci. Res.* 33:493-503, 1992; Baba et al., *J. Neurosurg.* 79:729-735, 1993 (U.S. Patent No. 4,777,127, GB 2,200,651, EP 345,242 and  
20 WO 91/02805). Preferred retroviral vectors include murine leukemia amphotropic or xenotropic, or VSV-G pseudotype vectors (*see*, WO 92/14829, incorporated herein by reference).

Retroviral gene delivery vehicles of the present invention may be readily constructed from a wide variety of retroviruses including, for example, B-, C-, and D-type  
25 retroviruses, as well as spumaviruses and lentiviruses (*see*, RNA Tumor Viruses, Second Edition, Cold Spring Harbor Laboratory, 1985). Briefly, viruses are often classified according to their morphology as seen under electron microscopy. Type "B" retroviruses appear to have an eccentric core, while type "C" retroviruses have a central core. Type "D" retroviruses have a morphology intermediate between type B and type C  
30 retroviruses. Representative examples of suitable retroviruses include a variety of xenotropic retroviruses (*e.g.*, NZB-X1, NZB-X2, and NZB<sub>9-1</sub> (*see*, O'Neill et al., *J. Vir.* 53:100-106, 1985)) and polytropic retroviruses (*e.g.*, MCF and MCF-MLV (*see*, Kelly et al., *J. Vir.* 45(1):291-298, 1983)). Such retroviruses may be readily obtained from depositories or collections such as the American Type Culture Collection ("ATCC";  
35 Rockville, Maryland), or isolated from known sources using commonly available techniques.

Gene delivery vehicles as used within the present invention refers to recombinant vehicles which contain nucleic acids that direct the expression on one or more heterologous nucleotide sequences, such as viral vectors (Jolly, *Cancer Gen. Therapy* 1:51-64, 1994). Representative examples of such gene delivery vehicles include poliovirus (Evans et al., *Nature* 339:385-388, 1989; and Sabin, *J. Biol. Standardization* 1:115-118, 1973); rhinovirus; pox viruses, such as canary pox virus or vaccinia virus (Fisher-Hoch et al., *PNAS* 86:317-321, 1989; Flexner et al., *Ann. N.Y. Acad. Sci.* 569:86-103, 1989; Flexner et al., *Vaccine* 8:17-21, 1990; U.S. Patent Nos. 4,603,112, 4,769,330, and 5,017,487; WO 89/01973); SV40 (Mulligan et al., *Nature* 277:108-114, 1979); retrovirus (U.S. Patent No. 4,777,127, GB 2,200,651, EP 0,345,242, and WO 91/02805); influenza virus (Luytjes et al., *Cell* 59:1107-1113, 1989; McMichael et al., *N. Eng. J. Med.* 309:13-17, 1983; and Yap et al., *Nature* 273:238-239, 1978); adenovirus (Berkner, *Biotechniques* 6:616-627, 1988; Rosenfeld et al., *Science* 252:431-434, 1991; WO 93/9191; Kolls et al., *PNAS* 91:215-219, 1994; Kass-Eisler et al., *PNAS* 90:11498-11502, 1993; Guzman et al., *Circulation* 88:2838-2848, 1993; Guzman et al., *Cir. Res.* 73:1202-1207, 1993; Zabner et al., *Cell* 75:207-216, 1993; Li et al., *Hum. Gene. Ther.* 4:403-409, 1993; Caillaud et al., *Eur. J. Neurosci.* 5:1287-1291, 1993; Vincent et al., *Nat. Genet.* 5:130-134, 1993; Jaffe et al., *Nat. Genet.* 1:372-378, 1992; and Levrero et al., *Gene* 101:195-202, 1991); parvovirus such as adeno-associated virus (Samulski et al., *J. Vir.* 63:3822-3828, 1989; Mendelson et al., *Virol.* 166:154-165, 1988; PA 7/222,684); herpes (Kit, *Adv. Exp. Med. Biol.* 215:219-236, 1989); SV40; HIV (Poznansky, *J. Virol.* 65:532-536, 1991); measles (EP 0 440,219); astrovirus (Munroe, S.S. et al., *J. Vir.* 67:3611-3614, 1993); Semliki Forest Virus, and coronavirus, as well as other viral systems (e.g., EP 0,440,219; WO 92/06693; U.S. Patent No. 5,166,057). In addition, viral carriers may be homologous, non-pathogenic (defective), replication competent virus (e.g., Overbaugh et al., *Science* 239:906-910,1988).

Within certain preferred embodiments, the gene delivery vehicle is a retrovirus. Retroviruses are RNA viruses with a single positive strand genome which, in general, are nonlytic. Upon infection, the retrovirus reverse transcribes its RNA genome into DNA, which is inserted into the host cell genome. Preparation of retroviral constructs for use in the present invention is described in greater detail in an application entitled "Recombinant Retroviruses" (U.S.S.N. 07/586,603, filed September 21, 1990), herein incorporated by reference. The retroviral genome can be divided conceptually into two parts. The "trans-acting" portion consists of the region coding for viral structural proteins, including the group specific antigen (*gag*) gene for synthesis of the core coat proteins; the *pol* gene for the synthesis of the reverse transcriptase and integrase enzymes; and the envelope (*env*) gene for the synthesis of envelope glycoproteins. The "cis-acting" portion

relative concentration of specific vector proteins, can be estimated based on the known number of copies per vector particle. Alternatively, the mass of the capsid p30 and related proteins can be determined by Western blots or ELISA in comparison to a standard. The number of particles can be calculated assuming  $3 \times 10^3$  of these molecules exist per particle. If the vector is formulated in a carrier protein such as human serum albumin (HSA), the molar ratio can be taken to be that of the carrier protein as it will also be modified.

The biological activity of the modified retroviral vectors can be determined by a variety of methods known to those of skill in the art. For example, the biological activity can be performed by measuring the relative infectivity in tissue culture with HT080 cells. In this procedure, blue colony forming unit (BCFU) titers are determined by counting wells for blue colonies after x-gal staining at two days post transduction. Titers can then be related to those of the unmodified vectors. (Current Protocols in Molecular Biology, Ausubel et al. eds.) Modification conditions are typically carefully determined on a trial basis in order to generate modified vector preparations with optimal retention of biological activity (at least 10-30% of the original activity).

Gene Delivery vehicles including retroviral vectors can be conjugated to large variety of polymers including dextran derivatives. Dextrans of low molecular weight are preferred, particularly dextrans of MW 1,000-10,000.

For example, polyaldehyde dextran can be prepared by  $\text{NaIO}_4$  oxidation according to the method of Bobbitt (*Adv. Carbohydrate Chem.* 11:1-43, 1956). This method results in the oxidization of the glucose rings with the generation of multiple aldehyde groups. Typically in this method, periodate oxidation proceeds for about 24 hours in the dark at room temperature. Oxidized dextrans can then dialyzed extensively and lyophilized. Polyaldehyde-dextrans can then be reacted with primary amines, such as the epsilon amino group of lysines of surface proteins of beta-gal vectors, to form Schiff bases, which can then stabilized by  $\text{NaCNBH}_3$  reduction. Residual aldehyde groups on protein-bound dextrans can be reduced with  $\text{NaBH}_4$ . The level of dextran substitution of beta-gal vectors can be measured by a variety of techniques such as an anthrone colorimetric assay, based on the determination of reducing sugars ( see Viles, F. J. Jr and Silverman, L., *Anal. Chem.* 21:950, 1949). This assay can also be used to determine the level of polyaldehyde content of periodate activated dextran.

Other derivatization conditions can be also be used, including the linkage of chain-terminal hydrazide-dextrans to the carbohydrate residues of surface proteins of vectors.



T. H. *Biochem Biophys Acta* 559:39, 1979). The photoactivation can be done by either exposing the reaction solution to direct light for 5-10 minutes or to 5-10 bright flashes. Cross-linkers containing phenyl azide require photoactivation in the 265-275 nm range, while those containing nitro-phenyl azide achieve optimal photolysis around 320-350 nm, which limits damage to biomolecules due to radiation (Schrock, A.K. and Schuster, G.B.J. *Am. Chem. Soc.* 106:5228, 1984).

Gene delivery vehicles including retroviral vectors can be derivatized with water soluble polymers to generate conjugates with the retention of biological properties and little or no immunogenicity. Examples of such polymers include, but are not limited to, polyethylene glycol (Peg) derivatives, dextrans of various molecular weights, ethylene-maleic anhydride copolymers, polyvinyl alcohols and others. Alternative derivatizations of retroviral vectors with water soluble polymers include; Peg-vinylsulfone, Peg-orthopyridyl-disulfide (OPSS-PEG), Peg-N-hydroxy-succinamide (PEG-NHS), Peg-Tresylate, Peg-allyl ether-maleic anhydride copolymer, and Peg-biotin.

For example, Peg-maleimide can be used to generate biologically active Peg-protein conjugates ( see Goodson, R.J. and Katre, N.V., *Biotechnology* 8:343, 1990; Kogan, T.P., *Synthetic Comm* 22:2417, 1992). Since this reaction requires free -SH groups, the protein to be derivatized may have to be treated with reducing agents in the case a free sulfhydryl is not available. Other techniques known to those of skill in the art can also be used to generate free -SH groups. For example, a free -SH group can be genetically engineered in a non-functional domain of the protein of interest to prevent potential reduction of biological activity resulting from the use of reducing agents. Retroviral vector preparations can be treated with optimal amounts of a reducing agent to generate free -SH groups under conditions that do not significantly affect vector activity. Ellman's reagent can be used to calculate the number of available sulfhydryls (Ellman, G.L., *Arch. Biochem. Biophys* 74:443, 1958).

Peg-maleimide is typically added to a solution of vector particles at molar ratios of 10-50 and a buffer pH of between 6.5 and 7.0. After incubation, Peg-derivatized vector particles can be purified from the reaction mixture using standard techniques such as gel filtration with Sephacryl S-400. Since derivatization reactions often proceed as a function of protein-to-polymer molar ratios, it may be useful to estimate the molar concentration of the candidate retroviral vector. This can be done, for example, by determining the number of vector particles mixed with polystyrene latex particles per unit volume by electron microscopy. In this procedure, the total number of vectors in a given preparation can be calculated based on the number of latex particles per unit of volume. Based on an estimated molecular weight of 200 million, the molar concentration of the vector can then be determined from the estimated molecular weight. From this, the

As is explained above, gene delivery vehicles including retroviral vector particles can be covalently modified for the purpose of reducing their immunogenicity with retention of significant biological activity. In addition, they can be derivatized via conjugation with specific tissue-targeting molecules to alter the targeting capabilities of these vectors. A variety of cross-linking agents can be used to derivatize proteins including heterobifunctional, homobifunctional, and photoreactive cross-linkers.

Heterobifunctional cross-linkers contain two reactive groups: a primary amine reactive group, usually N-Hydroxy-Succinimide (NHS) or its water-soluble derivative, sulfo-NHS, which reacts with primary amines such as the  $\epsilon$ -amino groups of lysine of proteins. The second reactive specificity of heterobifunctional cross-linkers is a thiol-reactive group, such as maleimides, halogens or pyridyl disulfides. By reacting with two distinct specificities, heterobifunctional cross-linking agents allow the linkage of two different proteins with significant reduction of unwanted side reactions like homoprotein polymers. Heterobifunctional cross-linking agents have been extensively used to covalently cross-link proteins to proteins, proteins to polymers, or polymers to polymers. A third component of a heterobifunctional cross-linking agent is the spacer arm, which connects the two reactive groups. The purpose of the spacer arm is primarily for steric hinderance and to provide added stability to the reactive groups of the cross-linking agent. Cross-linkers have spacer arms of varying lengths, as longer arms are at times required to effectively span the distance between two complex biomolecules and allow the reaction to take place.

Homobifunctional cross-linking agents possess two copies of the same functional reactive groups at both ends of a spacer arm. A majority of currently available homobifunctional cross-linkers are derivatives of either NHS or Sulfo-NHS esters while others are cross-linkers that do not contain NHS esters. The reaction conditions for NHS-based homobifunctional cross-linking agents are similar to the primary amine reaction of heterobifunctional cross-linkers. The NHS group reacts with primary amines such as the epsilon amino group of lysines or the N-terminus amine to form a stable amide bond with the release of N-hydroxy-succinimide as a by-product. This reaction is favored by neutral or alkaline pHs, where the primary amines are not protonated. They react by nucleophilic attack on NHS and by concentrated protein solutions, which favor the acylation reaction and reduce the inactivation of NHS by hydrolysis. By choosing a proper molar ratio of cross-linker to amine group, the extent of cross-linking can be varied to a desirable level.

Photoreactive heterobifunctional cross-linkers contain two different reactive sites, allowing the sequential conjugation of proteins with little or no polymerization or self-conjugation. The first step comprises the chemical linkage with the first protein to be conjugated. The linkage to the second protein then takes place under UV irradiation (Ji,

polysaccharides such as carboxy methylcellulose may also be used. Dextran is a preferred polysaccharide because of its widespread use as a plasma substitute in current clinical practice.

Alternatively, the compound to be conjugated may be a synthetic polymer, other than PEG. Suitable polymers include homopolymers and copolymers which have either terminal or pendant functional groups for coupling to a polycation. A polymer's pendant functional groups results from a functional group present in the monomer from which the polymer is derived. Suitable terminal or pendant functional groups include any functional group which is or may be converted to a reactive functional group for coupling to a polycation.

A homopolymer is a polymer composed of a single repeating unit and is derived from the polymerization of a single monomer. For example, polymerization of vinyl alcohol yields polyvinyl alcohol, a homopolymer consisting of a carbon backbone (*i.e.*, repeating methylene groups) and pendant hydroxy groups. In the practice of the present invention, the pendant hydroxy groups may be further functionalized by standard organic chemical synthetic techniques to provide appropriate functional groups for coupling to a polycation. For example, acylation with succinic (or glutaric) anhydride or alkylation with a 2-haloacetic acid derivative of the hydroxy groups of polyvinyl alcohol provide polymers with pendant carboxylic acid functional groups. The carboxylic acid groups of these polymers may be converted directly to reactive esters such as N-hydroxy succinimide esters for coupling. Polyacrylic acid is an example of another suitable homopolymer consisting of a carbon backbone and pendant carboxylic acid groups.

Copolymers are also suitable as molecules for conjugation. A copolymer is a polymer composed of more than a single repeating unit and is derived from more than one monomer. For example, copolymerization of two different monomers (*i.e.*, comonomers) such as ethylene and maleic anhydride yields an ethylene/maleic anhydride copolymer consisting of a carbon backbone and pendant functional groups (*i.e.*, anhydride groups). Such a copolymer may be coupled to a polycation through reaction with the pendant anhydride functional groups.

In a similar manner, gene delivery vehicles may be conjugated with polyalkylene glycol or polysaccharide. In general, conjugation is performed in similar manner to that described above. A reactive group is synthesized for polyalkylene glycol, preferably a NHS group, and for polysaccharide, preferably an aldehyde. Reaction conditions may need to be modified in a routine, minor fashion depending on the virus undergoing coupling.

tested in the immunogenicity test as described below. The preferred ratio is that which gives a statistically significant reduction in immunogenicity as compared to an unconjugated polycation.

As set forth above, in a separate aspect, polysaccharides may be conjugated to polycations to reduce their immunogenicity. Although many procedures well known in the art may be used to prepare a polysaccharide for conjugation, one such procedure is provided below for illustrative purposes, wherein polysaccharides are conjugated to polycations following oxidation with  $\text{NaIO}_4$ . Briefly, polysaccharide is dissolved in sodium borate buffer containing  $\text{NaIO}_4$  and stirred in the dark at room temperature for a day (Bobbitt, *Adv. Carbohydrate Chem.* 11:1, 1956). The reaction is terminated by the addition of a molar excess of ethylene glycol. Oxidized polysaccharide is dialyzed extensively against distilled water and subsequently lyophilized. Subsequent conjugation of oxidized polysaccharides to polycations is performed as follows. A solution of oxidized polysaccharide is added at a predetermined ratio to a solution of polycation in sodium phosphate buffer. A solution of  $\text{NaBH}_3\text{CN}$  in the same buffer is added at a large molar excess relative to polysaccharide, and the solution is stirred at room temperature for two hours. At the end of the incubation, dextran-conjugated polycation may be separated from unreacted polysaccharide by gel filtration. A relative aldehyde content of oxidized polysaccharide may be determined by an anthrone assay (Fagnani et al., *Cancer Res.* 50:3638-3645, 1990) or other suitable assay. Any free aldehyde groups remaining on the polycation dextran conjugate can be detrimental to the quality of the final product because these moieties can lead to the formation of undesirable high molecular weight aggregates, which tend to precipitate out of solution with time. To avoid this, the free aldehyde groups are reduced with a reducing agent, preferably  $\text{NaBH}_4$ . The extent of residual activation of oxidized dextran bound to the polycation may be determined by an anthrone/ $\text{H}_2\text{SO}_4$  method (Fagnani, *supra*). An amount of  $\text{NaBH}_4$  corresponding to the  $\text{NaBH}_4$ :oxidized dextran molar ratio producing greater than 80% reduction in color development is sufficient to adequately treat the polycation-dextran conjugates. Polysaccharide-conjugated polycations are then extensively dialyzed against sodium phosphate buffer. The optimal ratio of polysaccharide to polycation will depend upon the polycation used. The optimal ratio may be determined as described above.

Polysaccharides suitable for oxidation and subsequent conjugation to a polycation include homopolymers or heteropolymers of D-glucose, D-mannose, D-galactose, L-galactose, D-xylose, D-arabinose, D-glucosamine, D-glucuronic acid, N-acetyl-muramic acid and N-acetyl neuraminic acid. Natural polysaccharides that may be used include dextran, alpha-amylase, amylopectin, amylase-modified polysaccharides, fructans, such as insulin, mannans, xylans, and arabinans. Derivatives of these

oxidation. It will be recognized, however, that other reactive groups may be prepared by well-known chemical synthesis methods and used within the context of this invention.

As discussed above, in one embodiment, polycations are chemically conjugated with polyalkylene glycols. One example of such a polyalkylene glycol is polyethylene glycol (PEG). As an illustration of the invention, a model synthesis of conjugating PEG to polylysine is provided. Those in the art will recognize that other polyalkylene glycols and polycations, as well as alternative syntheses may readily be substituted. Briefly, the synthesis procedure first converts methoxy-PEG into a form amenable for conjugation, such as methoxypolyethylene glycol N-hydroxysuccinimidyl glutarate. Methoxy-PEG is conveniently used because it has only one hydroxyl group available for substitution. This compound may be prepared by the following procedure. PEG is first dissolved in toluene and distilled. The distilled PEG is reacted with glutaric anhydride in dicycloethane and dry pyridine to form PEG-glutarate. Following refluxing under nitrogen gas for three days, the mixture is filtered, and solvent is evaporated. The residue is dissolved in water and washed with diethyl ether. PEG-glutarate is then extracted from the water phase by chloroform washes. The chloroform is subsequently evaporated. PEG-glutarate is then dissolved in dimethylformamide and mixed with dicyclohexylcarbodiimide and N-hydroxysuccinimide in dimethylformamide and stirred vigorously. Upon the addition of benzene, methoxypolyethylene glycol N-hydroxysuccinimidyl glutarate (NHS-PEG) is precipitated by the dropwise addition of petroleum ether at 0°C. The precipitate is collected on a sintered glass filter, dissolved in benzene, and reprecipitated with petroleum ether. NHS-PEG may be stored at -20°C in a desiccator until use.

NHS-PEG may then be conjugated to the free  $\epsilon$ -amino groups of polylysine essentially as follows. Briefly, NHS-PEG and polylysine are stirred together at a predetermined ratio for 30 minutes. Any remaining activated ester is removed by reaction with an excess of  $\epsilon$ -aminocaproic acid. Unbound NHS-PEG is removed from the reaction mixture by size chromatography. The degree of amino group modification of the polycation may be determined by measuring the number of free amino groups with 2, 4, 6-trinitrobenzene sulphonic acid (TNBS) (Synder and Sobocinski, *Anal. Biochem.* 64:284-288, 1975) or NMR spectroscopy. The attachment of PEG to the polycation may also be confirmed by SDS-PAGE analysis or other suitable test. The optimum amount of PEG conjugation may be determined by the following two tests. The first test is to perform *in vitro* gene transfer and establish a ratio that reduces transfer efficiency of a reporter plasmid by about ten-fold. If this is not readily obtainable, a ratio near the maximum level of conjugation obtainable is chosen. Second, several conjugation ratios from zero to the near maximum ratio or the ratio that gives a ten-fold reduction in efficiency from the peak are

following the manufacturer's protocols. A complete bacterial high level expression and purification system known as Glutathione S-Transferase (GST) Gene Fusion System<sup>TM</sup> may be obtained from Pharmacia, Piscataway, NJ. Briefly, amplified DNA is cloned into the polylinker of one of the PGEX vectors from Pharmacia. The choice of PGEX vector is based on maintaining the desired reading frame for the cloned fragment and choosing either a factor Xa or thrombin protease recognition sequence as desired for cleavage during purification to remove the GST domain from the fusion protein. The vectors supply all necessary transcription and translation initiation sequences for high level inducible expression of either single or multiple units, obtained by simple sub-cloning procedures, of the desired basic domain. Fusion proteins containing the 26 kDa GST domain from *S. japonicum*, are purified from bacterial lysates by using the Bulk or RediPak GST Purification modules from Pharmacia. Cleavage of the desired amino acid domains from the fusion protein is achieved using a site specific protease, factor Xa or thrombin depending on the specific pGEX vector. All steps in expression and protein purification follow the manufacturer's protocols. Alternatively many other commercially available integrated expression/purification systems such as the "Flag" system from Kodak or other equivalent expression vector system may be used.

## 2. Conjugation of condensing agents

This invention provides polycations, useful as nucleic acid condensing agents, that have low immunogenicity. Polycations within the context of this invention include polylysine, polyarginine, histones, protamines, spermidine, spermine, and other highly basic proteins or polypeptides. In one embodiment, the polycation is chemically conjugated with one or more polyalkylene glycol. In one aspect of the invention, the polyalkylene glycol is polyethylene glycol. In another embodiment, the polycation is conjugated with one or more polysaccharides. Briefly, chemical conjugation involves forming a covalent linkage between the polycation and the polyalkylene glycol or polysaccharide. The conjugation may be performed either prior to or following condensation of the nucleic acid with the polycation. If conjugation is performed after condensation, other components discussed below, such as ligand molecules, may be present during the conjugation procedure. Many suitable methods for forming the linkage may be determined by one skilled in the art given the disclosure provided herein. In general, these methods first prepare the polyalkylene glycol or polysaccharide for coupling by creating an active group in place of a terminal OH group. A preferred reactive group for polyalkylene glycols is N-hydroxy succinimide. A preferred reactive group for polysaccharides is an aldehyde, which is present on some natural sugars or may be generated by chemical

of viable virus, as well as one or more restriction sites. When the Sindbis vector genome is transfected as a DNA molecule to give a eucaryotic layered vector system (ELVS), it should additionally include a 5' RNA polymerase II promoter which is capable of initiating the synthesis of viral RNA from cDNA, and a 3' sequence which controls transcription termination and splice recognition.

#### CONDENSING AGENTS

Nucleic acid condensing agents facilitate the transport of DNA or other nucleic acids into a cell. Such agents should not themselves be immunogenic or the efficiency of gene transfer will be diminished. This invention provides nucleic acid condensing agents of low immunogenicity comprising polycations conjugated with molecules and basic amino acid regions generally derived from proteins native to the species which is the target of gene transfer.

In general, to obtain a condensing agent within the context of this invention, the following strategy is employed. First, the condensing agent is prepared. Polycations are chemically conjugated with polyalkylene glycols, polysaccharides, derivatives, or similar compounds. A second type of condensing agent, novel condensing agents, comprising basic amino acid regions, are identified by interrogation of protein databases. The candidate regions are then chemically or biologically synthesized.

20

##### 1. Preparation of polycations

Homopolymers of polylysine may be obtained from Sigma (St. Louis, MO) (e.g., product #2636). Similarly, other homopolymers of several other polycations having various average lengths, such as polyarginine and polyornithine, may also be obtained from Sigma. Histone proteins, including H1, H3, and H4, may be obtained from Boehringer Mannheim (Indianapolis, Indiana). Spermine or spermidine, as well as protamine chloride, may be obtained from Sigma.

Preparation of these polycations and those which are derived from basic amino acid regions of a suitably identified protein may be synthesized by standard chemical techniques in lengths up to approximately 50 amino acids. Alternatively, the corresponding region of the gene may be isolated by conventional techniques such as PCR amplification using primers containing restriction sites chosen for convenience. Such amplified fragments may then be cloned and expressed in any of a variety of commercially available expression vectors designed for constitutive or transient expression in bacteria, yeast, or other eukaryotic cells.

As one such example, an amplified nucleic acid of interest is cloned into a bacterial expression system and the resulting polypeptide is expressed and purified

of this invention, these complexes can be administered multiple times to the same host without eliciting unfavorable immune responses.

"Polyalkylene glycols" refer to 2 or 3 carbon polymers of glycols. Two carbon polyalkylenes include polyethylene glycol (PEG) of various molecular weights, and  
5 its derivatives, such as polysorbate, polyoxyethylene sorbitan monolaurate, and polyethylene glycol-*p*-isooctylphenyl ether. Three carbon polyalkylenes include polypropylene glycol and its derivatives.

"Polysaccharides" refer to polymers of three or more monomeric sugars. These sugars include D-glucose, D-mannose, D-fructose, D-galactose, L-galactose, D-  
10 xylose and D-arabinose. In addition, derivative monosaccharides may also be polymerized. Such derivatives include D-glucosamine, D-glucuronic acid, N-acetylmuramic acid, and N-acetyl neuraminic acid. Polymers of these monosaccharides may be composed of one type of saccharide or multiple types of saccharides and may be various molecular weights. Naturally occurring polysaccharides that may be used within the scope of this invention  
15 include dextrans,  $\alpha$ -amylose, amylopectin, amylase-modified versions of polysaccharides, fructans, mannans, xylans, and arabinans.

"Nucleic acid" refers to DNA, RNA, analogues thereof, peptide-nucleic acids, and DNA or RNA with non-phosphate containing nucleotides. Additionally, these nucleic acids may be single-stranded, double-stranded, or chimeric single- or double-  
20 stranded molecules.

"Gene delivery vehicle" refers to an assembly which is capable of directing the expression of sequence(s) or gene(s) of interest. The gene delivery vehicle will generally include promoter elements and may include a signal that directs polyadenylation. In addition, the gene delivery vehicle includes a sequence which, when transcribed, is  
25 operably linked to the sequence(s) or gene(s) of interest and acts as a translation initiation sequence. The gene delivery vehicle may also include a selectable marker such as *neo*, TK, hygromycin, phleomycin, histidinol, or DHFR, as well as one or more restriction sites and a translation termination sequence. In addition, if the gene delivery vehicle is a retrovirus, a packaging signal and long terminal repeats (LTRs) appropriate to the retrovirus used will  
30 be included (if these are not already present). Further, if the gene delivery vehicle is a Sindbis virus, the vehicle RNA will include a 5' sequence which is capable of initiating transcription, as well as sequences which, when expressed, code for biologically active Sindbis non-structural proteins (*i.e.*, NS1, NS2, NS3, and NS4). In addition, the Sindbis gene delivery vehicle should include a Sindbis RNA polymerase recognition sequence, and  
35 a viral junction region, which may, in certain embodiments, be modified in order to either prevent or inhibit viral transcription of the subgenomic fragment. The gene delivery vehicle may also include nucleic acid molecule(s) which are of a size sufficient to allow production



### Detailed Description of the Invention

Prior to setting forth the invention, it may be helpful to an understanding thereof to set forth definitions of certain terms that will be used hereinafter.

5 "Nucleic acid condensing agent" refers to a compound, natural or synthetically produced, which when combined with nucleic acids causes it to assume a condensed structure relative to uncomplexed nucleic acids. Condensation of DNA molecules to a size that may be internalized by coated pit structures present on cell surface membranes is preferable.

10 "Polycation" refers generally to a polymer of positively-charged single units, although some non-positively charged units may be present in the polymer. Preferably, for polycations that are polypeptides, they should have a predicted isoelectric point of at least 9 and contain at least 40% basic residues (generally, lysine plus arginine). Additionally, there should not be greater than 5% acidic residues and preferably none. Examples of polycations include polylysine, polyarginine, polyornithine, spermine, spermidine, 15 putrescine, and protamine. For polycations composed of amino acids, either the L- or D-forms may be used. Basic amino acids include lysine, arginine, amino acid analogues such as ornithine and canaline, modifications of basic amino acids, such as homoarginine, and modifications of other amino acids such as to carry a positive charge, such as guanidinovalinate, and aminoethylcysteine. As a general rule, a basic amino acid has a pK 20 value for the side chain of at least 7.5.

"Basic amino acid region" refers to domains of 50 to 300 amino acids in length that have a predicted isoelectric point of at least 9, and at least 40% basic residues. In most proteins, these residues are generally arginine and lysine. In addition, there should be not greater than 5% acidic amino acid residues. Basic amino acid regions may be 25 derived from known proteins, including DNA binding proteins, such as histones and transcription factors, coat proteins from DNA viruses, or be synthetically derived.

"Immunogenicity" refers to the ability of a given molecule or a determinant thereof to induce the generation of antibodies with binding capacity to the molecule upon administration *in vivo*, to induce a cytotoxic response, activate the complement system, 30 induce allergic reactions, and the like. An immune response may be measured by assays that determine the level of specific antibodies in serum, by assays that determine the presence of a serum component that inactivates the condensing agent/nucleic acid complex or conjugated gene delivery vehicle, or by other assays that measure a specific component or activity of the immune system. As discussed in more detail below, low immunogenicity 35 may be established by these assays. The terms "low immunogenicity," "reduced immunogenicity," "lowered immunogenicity" or similar terms are intended to be equivalent terms. Because of the low immunogenicity of the nucleic acid/condensing agent complexes

molecular weight ranging from 1,000 to 90,000. In other embodiments of the invention, the gene delivery vehicle further comprises a pharmaceutically acceptable carrier. In yet other embodiments, the gene delivery vehicle further comprises a ligand capable of targeting the gene delivery vehicle to a selected cell type.

5           Within still other aspects of the invention, nucleic acids condensed with a nucleic acid condensing agent comprising a basic amino acid region of at least 50 amino acids, in which the region contains at least 40% basic amino acids, has a predicted isoelectric point of at least 9, and contains not greater than 5% acidic amino acids are provided. In addition, the combination of nucleic acid condensing agent and nucleic acids  
10           exhibits low immunogenicity. The basic amino acid region may be derived from a protein, such as human serum albumin, histone, DNA binding proteins, protamines and/or non-histone chromosomal proteins. In one embodiment of this aspect, a plurality or multiplicity of basic amino acid regions are linked in a tandem array. In a related embodiment, the array contains between 1-10 basic amino acid regions. In other embodiments of the  
15           invention, the nucleic acids further comprise a pharmaceutically acceptable carrier and a ligand capable of targeting the nucleic acids to a selected cell type.

          In another aspect, methods of gene transfer in a patient are provided, comprising the step of administering to a patient nucleic acids condensed with a nucleic acid condensing agent comprising a polycation chemically conjugated either with  
20           polyalkylene glycol or polysaccharide, a gene delivery vehicle chemically conjugated with polyalkylene glycol or polysaccharide, nucleic acids condensed with a nucleic acid condensing agent comprising a basic amino acid region. These nucleic acids or gene delivery vehicles are additionally in a carrier pharmaceutically acceptable to a patient. In  
25           embodiments of this method, a ligand, which is capable of targeting the nucleic acids to a selected cell type is also provided. In yet other embodiments, the nucleic acids encode a protein, are transcribed into other nucleic acids, or themselves are capable of stimulating an immune response, suppressing an immune response, encoding a prodrug, encoding a cytokine, which is administered to a tumor, or capable of constitutive production of  
30           proteins.

          These and other aspects of the invention will become evident upon reference upon the following detailed description. In addition, various references are set forth below which describe in more detail certain procedures or compositions. Each of these references are incorporated herein by reference in their entirety as if each were individually noted for  
35           incorporation.

foreign and an immune response is generated. Thus, the usefulness of these vectors for multiple administrations is limited. Initial administration may also elicit an undesirable inflammatory response if the individual had prior exposure to wild-type virus (*See, Jolly, Cancer Gene Therapy 1:51, 1994*).

5           In view of the problems associated with current gene delivery mechanisms, there is a compelling need for polycations which are equally or more effective and are not associated with the disadvantage of being immunogenic. The present invention provides compositions and methods for the use of compounds conjugated to polycations and gene delivery vehicles that reduce immunogenicity and novel condensing agents with reduced  
10 immunogenicity, as well as other, related advantages.

#### Summary of the Invention

The present invention generally provides nucleic acid condensing agents in which the nucleic acid condensing agent, in combination with nucleic acids, exhibits low or  
15 negligible immunogenicity. In one aspect, nucleic acid condensing agents are provided, comprising a polycation chemically conjugated with polyalkylene glycol. In a preferred embodiment, the polyalkylene glycol is polyethylene glycol. It is preferred that the polyethylene glycol has a molecular weight of 200 to 10,000. In a second aspect, nucleic acids are condensed with a nucleic acid condensing agent comprising a polycation  
20 chemically conjugated with polysaccharide. In a preferred embodiment of this aspect, the polysaccharide is dextran. In a further embodiment, the dextran has a molecular weight in the range 1,000 to 90,000. In embodiments of the invention, the polycation is selected from the group consisting of polylysine, protamines, histones, spermine, spermidine, polyornithine, polyarginine, and putrescine. In other embodiments, the nucleic acids further  
25 comprise a pharmaceutically acceptable carrier. In yet other embodiments, a ligand capable of targeting the nucleic acids to a selected cell type is included. Representative examples of such ligands include transferrin, asialoglycoprotein, antibody, antibody fragments, low density lipoproteins, interleukins, GM-CSF, G-CSF, M-CSF, stem cell factor and erythropoietin.

30           In another aspect, gene delivery vehicles are provided that are chemically conjugated with polyalkylene glycol, such that the conjugated product exhibits low immunogenicity. In one embodiment of this aspect, the polyalkylene glycol is polyethylene glycol. It is preferred that the polyethylene glycol has a molecular weight ranging from 200 to 10,000. In a related aspect, gene delivery vehicles are chemically conjugated with a  
35 polysaccharide, such that the conjugated product exhibits low immunogenicity. In one embodiment, the polysaccharide is dextran. In a further embodiment, dextran has a

## NUCLEIC ACID CONDENSING AGENTS WITH REDUCED IMMUNOGENICITY

### Technical Field

5                   This application relates to nucleic acid condensing agents, and in particular, to agents with reduced immunogenicity that are useful for gene transfer *in vivo*.

### Background of the Invention

10                   Successful gene transfer into animals or humans requires a gene delivery vehicle that can transport genetic information into target cells for expression of desired therapeutic proteins. In general, such gene delivery vehicles are either virus vectors, notably based on retroviruses, adenoviruses and vaccinia viruses, or non-viral vectors, which utilize a physical gene transfer mechanism.

15                   Nucleic acids, typically DNA, delivered by a physical gene transfer mechanism is usually delivered by a receptor-mediated endocytosis pathway, a cellular mechanism which internalizes specific macromolecules. In general, complexes designed to be delivered in this fashion contain DNA encoding the gene of interest and a polycation which acts as a DNA binding domain and both neutralizes the charge on DNA and condenses the DNA. Condensation facilitates entry of DNA into cell vesicle systems by  
20                   simulating a macromolecular structure. Optionally, the complex includes a ligand which directs the complex to particular cells expressing the ligand-binding partner, and an endosomolytic agent, which serves to cause disruption of the endosome containing the complex.

25                   *In vivo* delivery of DNA by physical gene transfer has resulted in the successful expression of human serum albumin in rats (Wu et al., *J. Biol. Chem.* 266:14338, 1991) and luciferase gene to the airway epithelium of cotton rats (Gau et al., *Hum. Gene Ther.* 4:17-24, 1993). However, the efficiency of gene transfer in these systems was less than predicted from *in vitro* tests. Subsequently, it has been shown that such DNA complexes are unstable *in vivo*. Specifically, the polylysine component used in  
30                   these complexes is thought to be immunogenic, eliciting a humoral immune response, and may additionally or alternatively be inactivated by a serum component, probably a protein in the complement pathway. This instability reduces the overall efficiency of gene transfer *in vivo*.

35                   The other commonly employed gene transfer mechanism, viral vectors, are typically foreign agents to the host. The proteins of the viral capsids may be recognized as

*FOR THE PURPOSES OF INFORMATION ONLY*

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AM	Armenia	GB	United Kingdom	MW	Malawi
AT	Austria	GE	Georgia	MX	Mexico
AU	Australia	GN	Guinea	NE	Niger
BB	Barbados	GR	Greece	NL	Netherlands
BE	Belgium	HU	Hungary	NO	Norway
BF	Burkina Faso	IE	Ireland	NZ	New Zealand
BG	Bulgaria	IT	Italy	PL	Poland
BJ	Benin	JP	Japan	PT	Portugal
BR	Brazil	KE	Kenya	RO	Romania
BY	Belarus	KG	Kyrgyzstan	RU	Russian Federation
CA	Canada	KP	Democratic People's Republic of Korea	SD	Sudan
CF	Central African Republic	KR	Republic of Korea	SE	Sweden
CG	Congo	KZ	Kazakhstan	SG	Singapore
CH	Switzerland	LI	Liechtenstein	SI	Slovenia
CI	Côte d'Ivoire	LK	Sri Lanka	SK	Slovakia
CM	Cameroon	LR	Liberia	SN	Senegal
CN	China	LT	Lithuania	SZ	Swaziland
CS	Czechoslovakia	LU	Luxembourg	TD	Chad
CZ	Czech Republic	LV	Latvia	TG	Togo
DE	Germany	MC	Monaco	TJ	Tajikistan
DK	Denmark	MD	Republic of Moldova	TT	Trinidad and Tobago
EE	Estonia	MG	Madagascar	UA	Ukraine
ES	Spain	ML	Mali	UG	Uganda
FI	Finland	MN	Mongolia	US	United States of America
FR	France	MR	Mauritania	UZ	Uzbekistan
GA	Gabon			VN	Viet Nam



## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

<b>(51) International Patent Classification <sup>6</sup> :</b> <b>C12N 15/87, A61K 47/87</b>	<b>A2</b>	<b>(11) International Publication Number:</b> <b>WO 96/21036</b> <b>(43) International Publication Date:</b> 11 July 1996 (11.07.96)
<b>(21) International Application Number:</b> PCT/US95/17005 <b>(22) International Filing Date:</b> 26 December 1995 (26.12.95)  <b>(30) Priority Data:</b> 08/366,787 30 December 1994 (30.12.94) US  <b>(71) Applicant:</b> CHIRON VIAGENE, INC. [US/US]; 4560 Horton Street, Emeryville, CA 94608 (US). <b>(72) Inventors:</b> DE POLO, Nicholas, J.; 567 Hygeia Avenue, Leucadia, CA 92024 (US). HSU, David, Chi-Tang; 8012 Camino Tranquila, San Diego, CA 92122 (US). <b>(74) Agents:</b> KRUSE, Norman, J. et al.; Chiron Corporation, Intellectual Property - R440, P.O. Box 8097, Emeryville, CA 94662-8097 (US).		<b>(81) Designated States:</b> AM, AT, AU, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IS, JP, KE, KG, KP, KR, KZ, LK, LR, LT, LU, LV, MD, MG, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TT, UA, UG, UZ, VN, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).  <b>Published</b> <i>Without international search report and to be republished upon receipt of that report.</i>
<b>(54) Title:</b> NUCLEIC ACID CONDENSING AGENTS WITH REDUCED IMMUNOGENICITY  <b>(57) Abstract</b> <p>Nucleic acid condensing agents with reduced immunogenicity are generated either by conjugation of polycations or by selection of basic amino acid regions from proteins. Conjugation involves a chemical linkage between a polyalkylene glycol, such as polyethylene glycol, or a polysaccharide, such as dextran, and a polycation. Additionally, gene delivery vehicles, such as viral vectors, may be conjugated with polyalkylene glycol or polysaccharide, to reduce their immunogenicity. Basic amino acid regions of proteins are identified by isoelectric point, and amino acid composition. These condensing agents are complexed with nucleic acids and used to deliver genes to cells. Immunogenicity is assessed by whether neutralizing antibody is induced and by whether a serum component inactivates the complexes.</p>		

# TRAITE DE COOPERATION EN MATIERE DE BREVETS

## PCT

### RAPPORT DE RECHERCHE INTERNATIONALE

(article 18 et règles 43 et 44 du PCT)

Référence du dossier du déposant ou du mandataire <b>ST98046</b>	<b>POUR SUITE A DONNER</b> voir la notification de transmission du rapport de recherche internationale (formulaire PCT/ISA/220) et, le cas échéant, le point 5 ci-après	
Demande internationale n° <b>PCT/FR 99/ 02995</b>	Date du dépôt international(jour/mois/année) <b>02/12/1999</b>	(Date de priorité (la plus ancienne) (jour/mois/année) <b>03/12/1998</b>
Déposant <b>AVENTIS PHARMA S.A</b>		

Le présent rapport de recherche internationale, établi par l'administration chargée de la recherche internationale, est transmis au déposant conformément à l'article 18. Une copie en est transmise au Bureau international.

Ce rapport de recherche internationale comprend 4 feuilles.

☒ Il est aussi accompagné d'une copie de chaque document relatif à l'état de la technique qui y est cité.

**1. Base du rapport**

- a. En ce qui concerne la **langue**, la recherche internationale a été effectuée sur la base de la demande internationale dans la langue dans laquelle elle a été déposée, sauf indication contraire donnée sous le même point.
- ☐ la recherche internationale a été effectuée sur la base d'une traduction de la demande internationale remise à l'administration.
- b. En ce qui concerne **les séquences de nucléotides ou d'acides aminés** divulguées dans la demande internationale (le cas échéant), la recherche internationale a été effectuée sur la base du listage des séquences :
- ☐ contenu dans la demande internationale, sous forme écrite.
- ☐ déposée avec la demande internationale, sous forme déchiffrable par ordinateur.
- ☐ remis ultérieurement à l'administration, sous forme écrite.
- ☐ remis ultérieurement à l'administration, sous forme déchiffrable par ordinateur.
- ☐ La déclaration, selon laquelle le listage des séquences présenté par écrit et fourni ultérieurement ne vas pas au-delà de la divulgation faite dans la demande telle que déposée, a été fournie.
- ☐ La déclaration, selon laquelle les informations enregistrées sous forme déchiffrable par ordinateur sont identiques à celles du listage des séquences présenté par écrit, a été fournie.

2. ☒ **Il a été estimé que certaines revendications ne pouvaient pas faire l'objet d'une recherche** (voir le cadre I).
3. ☐ **Il y a absence d'unité de l'invention** (voir le cadre II).

**4. En ce qui concerne le titre,**

- ☒ le texte est approuvé tel qu'il a été remis par le déposant.
- ☐ Le texte a été établi par l'administration et a la teneur suivante:

**5. En ce qui concerne l'abrégé,**

- ☒ le texte est approuvé tel qu'il a été remis par le déposant
- ☐ le texte (reproduit dans le cadre III) a été établi par l'administration conformément à la règle 38.2b). Le déposant peut présenter des observations à l'administration dans un délai d'un mois à compter de la date d'expédition du présent rapport de recherche internationale.

**6. La figure des dessins à publier avec l'abrégé est la Figure n°**

- ☐ suggérée par le déposant.
- ☐ parce que le déposant n'a pas suggéré de figure.
- ☐ parce que cette figure caractérise mieux l'invention.

☒ Aucune des figures n'est à publier.





# RAPPORT DE RECHERCHE INTERNATIONALE

Demande Internationale No

PCT/FR 99/02995

**A. CLASSEMENT DE L'OBJET DE LA DEMANDE**  
CIB 7 C12N15/87 A61K48/00 C07H15/04

Selon la classification internationale des brevets (CIB) ou à la fois selon la classification nationale et la CIB

**B. DOMAINES SUR LESQUELS LA RECHERCHE A PORTE**

Documentation minimale consultée (système de classification suivi des symboles de classement)  
CIB 7 A61K

Documentation consultée autre que la documentation minimale dans la mesure où ces documents relèvent des domaines sur lesquels a porté la recherche

Base de données électronique consultée au cours de la recherche internationale (nom de la base de données, et si réalisable, termes de recherche utilisés)

MEDLINE, BIOSIS, CHEM ABS Data

**C. DOCUMENTS CONSIDERES COMME PERTINENTS**

Catégorie *	Identification des documents cités, avec, le cas échéant, l'indication des passages pertinents	no. des revendications visées
X	<p>FUHRHOP J -H ET AL: "BOLAAMPHIPHILES WITH MANNOSE- AND TETRAALKYLAMMONIUM HEAD GROUPS ASCOATINGS FOR NUCLEIC ACIDS AND POSSIBLE REAGENTS FOR TRANSFECTIONS" CHEMISTRY AND PHYSICS OF LIPIDS, vol. 43, 1 avril 1987 (1987-04-01), pages 193-213, XP000562618 ISSN: 0009-3084</p>	<p>1-5, 8-12, 18-22, 26,28-33</p>
Y	<p>le document en entier</p>	<p>23-25,27</p>
Y	<p>WO 96 25508 A (RHONE POULENC RORER SA ;BYK GERARDO (FR); SCHERMAN DANIEL (FR); SC) 22 août 1996 (1996-08-22) cité dans la demande le document en entier</p>	<p>23-25,27</p>
	-/--	

☒ Voir la suite du cadre C pour la fin de la liste des documents

☒ Les documents de familles de brevets sont indiqués en annexe

\* Catégories spéciales de documents cités:

"A" document définissant l'état général de la technique, non considéré comme particulièrement pertinent

"E" document antérieur, mais publié à la date de dépôt international ou après cette date

"L" document pouvant jeter un doute sur une revendication de priorité ou cité pour déterminer la date de publication d'une autre citation ou pour une raison spéciale (telle qu'indiquée)

"O" document se référant à une divulgation orale, à un usage, à une exposition ou tous autres moyens

"P" document publié avant la date de dépôt international, mais postérieurement à la date de priorité revendiquée

"T" document ultérieur publié après la date de dépôt international ou la date de priorité et n'appartenant pas à l'état de la technique pertinent, mais cité pour comprendre le principe ou la théorie constituant la base de l'invention

"X" document particulièrement pertinent; l'invention revendiquée ne peut être considérée comme nouvelle ou comme impliquant une activité inventive par rapport au document considéré isolément

"Y" document particulièrement pertinent; l'invention revendiquée ne peut être considérée comme impliquant une activité inventive lorsque le document est associé à un ou plusieurs autres documents de même nature, cette combinaison étant évidente pour une personne du métier

"&" document qui fait partie de la même famille de brevets

Date à laquelle la recherche internationale a été effectivement achevée

30 juin 2000

Date d'expédition du présent rapport de recherche internationale

06/07/2000

Nom et adresse postale de l'administration chargée de la recherche internationale

Office Européen des Brevets, P.B. 5818 Patentlaan 2  
NL - 2280 HV Rijswijk  
Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,  
Fax: (+31-70) 340-3016

Fonctionnaire autorisé

Sitch, W



## C.(suite) DOCUMENTS CONSIDERES COMME PERTINENTS

Catégorie	Identification des documents cités, avec, le cas échéant, l'indication des passages pertinents	no. des revendications visées
A	<p>W0 98 06869 A (PHILADELPHIA CHILDREN HOSPITAL) 19 février 1998 (1998-02-19)  page 5, ligne 26 -page 10, ligne 30  page 16, ligne 7 -page 35, ligne 15  ---</p>	
A	<p>W0 96 21036 A (VIAGENE INC)  11 juillet 1996 (1996-07-11)  page 2, ligne 13 -page 3, ligne 29  page 5, ligne 8 - ligne 16  ---</p>	
A	<p>DATABASE CHEMICAL ABSTRACTS 'en ligne!  abstract no. 126:207866,  ERBACHER ET AL: "THE REDUCTION OF THE  POSITIVE CHARGES OF POLYLYSINE BY PARTIAL  GLUCONOYLATION INCREASES THE TRANSFECTION  EFFICIENCY OF POLYLYSINE/DNA COMPLEXES"  XP002116398  abrégé  &amp; BIOCHIM. BIOPHYS. ACTA,  vol. 1324, no. 1, 1997, pages 27-36,  ---</p>	
A	<p>DATABASE MEDLINE 'en ligne!  US NATIONAL LIBRARY OF MEDICINE (NLM),  BETHESDA, MD, US;  abstract 1999129193,  GOULA ET AL: "POLYETHYLENIMINE-BASED  INTRAVENOUS DELIVERY OF TRANSGENES TO  MOUSE LUNG"  XP002116399  abrégé  &amp; GENE THERAPY,  vol. 5, no. 9, septembre 1998 (1998-09),  pages 1291-1295,  ---</p>	
A	<p>DATABASE MEDLINE 'en ligne!  US NATIONAL LIBRARY OF MEDICINE (NLM),  BETHESDA, MD, US;  ABSTRACT 96200295,  OKU ET AL: "EFFECT OF SERUM PROTEIN  BINDING ON REAL-TIME TRAFFICKING OF  LIPOSOMES WITH DIFFERENT CHARGES ANALYZED  BY POSITRON EMISSION TOMOGRAPHY"  XP002116400  abrégé  &amp; BIOCHIMICA ET BIOPHYSICA ACTA,  vol. 1280, no. 1,  3 avril 1996 (1996-04-03), pages 149-154,  -----</p>	



# INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/FR 99/02995

Patent document cited in search report		Publication date	Patent family member(s)	Publication date
W0 9625508	A	22-08-1996	FR 2730637 A	23-08-1996
			AU 706643 B	17-06-1999
			AU 4835396 A	04-09-1996
			BR 9607383 A	25-11-1997
			CA 2211162 A	22-08-1996
			CZ 9702592 A	12-11-1997
			EP 0809705 A	03-12-1997
			FI 973363 A	15-08-1997
			HU 9801207 A	28-08-1998
			JP 11500431 T	12-01-1999
			NO 973745 A	14-08-1997
			SK 111897 A	04-02-1998
			US 5945400 A	31-08-1999
			ZA 9601255 A	27-08-1996
W0 9806869	A	19-02-1998	US 5948681 A	07-09-1999
			AU 4065997 A	06-03-1998
W0 9621036	A	11-07-1996	AU 4690596 A	24-07-1996



## TRAITE DE COOPERATION EN MATIERE DE BREVETS

PCT

## NOTIFICATION D'ELECTION

(règle 61.2 du PCT)

Expéditeur: le BUREAU INTERNATIONAL

Destinataire:

Assistant Commissioner for Patents  
United States Patent and Trademark  
Office  
Box PCT  
Washington, D.C.20231  
ETATS-UNIS D'AMERIQUE

en sa qualité d'office élu

Date d'expédition (jour/mois/année) 11 juillet 2000 (11.07.00)	
Demande internationale no PCT/FR99/02995	Référence du dossier du déposant ou du mandataire ST98046
Date du dépôt international (jour/mois/année) 02 décembre 1999 (02.12.99)	Date de priorité (jour/mois/année) 03 décembre 1998 (03.12.98)
Déposant HERSCOVICI, Jean etc	

1. L'office désigné est avisé de son élection qui a été faite:

☒ dans la demande d'examen préliminaire international présentée à l'administration chargée de l'examen préliminaire international le:

07 juin 2000 (07.06.00)

☐ dans une déclaration visant une élection ultérieure déposée auprès du Bureau international le:

2. L'élection ☒ a été faite

☐ n'a pas été faite

avant l'expiration d'un délai de 19 mois à compter de la date de priorité ou, lorsque la règle 32 s'applique, dans le délai visé à la règle 32.2b).

Bureau international de l'OMPI 34, chemin des Colombettes 1211 Genève 20, Suisse	Fonctionnaire autorisé Diana Nissen
no de télécopieur: (41-22) 740.14.35	no de téléphone: (41-22) 338.83.38





NOUVEAUX AGENTS DE TRANSFERT D'ACIDES NUCLEIQUES,  
COMPOSITIONS LES CONTENANT ET LEURS UTILISATIONS

La présente invention se rapporte à de nouveaux agents de transfert, les compositions les contenant et leurs utilisations pour le transfert *in vitro*, *in vivo* ou *ex vivo* d'acides nucléiques dans les cellules.

Avec le développement des biotechnologies, la possibilité de transférer efficacement des acides nucléiques dans les cellules est devenue une technique de base avec de nombreuses applications biotechnologiques. Il peut s'agir du transfert d'acides nucléiques dans des cellules *in vitro*, par exemple pour la production de protéines recombinantes, ou au laboratoire pour l'étude de la régulation de l'expression des gènes, le clonage de gènes ou tout autre manipulation impliquant l'ADN. Il peut également s'agir du transfert d'acides nucléiques dans des cellules *in vivo*, par exemple pour la réalisation de vaccins, des études de marquage ou également des approches thérapeutiques. Il peut encore s'agir du transfert de gènes dans des cellules prélevées d'un organisme, en vue de leur réadministration ultérieure, par exemple pour la création d'animaux transgéniques.

Actuellement, le moyen le plus répandu pour transférer des gènes dans des cellules est l'utilisation de vecteurs viraux. Mais ceux-ci n'étant pas complètement dénués de risques, plusieurs autres méthodes basées sur l'emploi de vecteurs synthétiques ont été proposées. Ces vecteurs synthétiques ont deux fonctions principales : complexer et compacter l'acide nucléique à transférer, et promouvoir son passage à travers la membrane plasmique et éventuellement à travers les deux membranes nucléaires.

Plusieurs familles de vecteurs synthétiques ont été développées, comme par exemple les polymères ou encore les vecteurs biochimiques (constitués d'une protéine cationique associée à un récepteur cellulaire), mais un progrès important a surtout été accompli en transfection non-virale avec le développement des lipofectants, et plus particulièrement des lipides cationiques. Il a ainsi été mis en évidence que les lipides

cationiques, du fait de leur charge globale positive, interféraient spontanément avec l'ADN globalement négatif, formant des complexes nucléolipidiques capables de fusionner avec les membranes cellulaires, et permettaient ainsi la libération intracellulaire de l'ADN.

5            Différentes sortes de lipides cationiques ont ainsi été synthétisés : des lipides comportant un groupement ammonium quaternaire (par exemple le DOTMA, DOTAP, DMRIE, DLRIE...), des lipopolyamines comme par exemple le DOGS, le DC-Chol ou encore les lipopolyamines divulguées dans la demande de brevet  
10            WO 97/18185, des lipides associant à la fois un groupement ammonium quaternaire et une polyamine comme le DOSPA, ou encore des lipides comportant diverses autres entités cationiques, notamment des groupes amidinium (par exemple l'ADPDE, ADODE ou les lipides de la demande de brevet WO 97/31935). En fait, la diversité structurelle des lipides cationiques reflète en partie l'observation de la relation structure-activité.

15            Toutefois, l'emploi de ces vecteurs synthétiques pose encore de nombreuses difficultés, et leur efficacité reste à améliorer. Notamment, il serait souhaitable de pouvoir disposer de vecteurs non-cationiques ou moins cationiques , et ce pour différentes raisons :

- 20            - les complexes formés entre l'acide nucléique et les agents de transfert, du fait de leur charge globale positive, ont tendance à être captés par le système réticuloendothélial ce qui induit leur élimination,
- du fait de la charge globale positive des complexes formés, les protéines du plasma ont tendance à s'adsorber à leur surface, et il en résulte une perte du pouvoir de transfection,
- 25            - dans un contexte d'injection locale, la présence d'une charge globale positive importante empêche la diffusion des complexes d'acides nucléiques en dehors du site d'administration , car les complexes s'adsorbent sur les matrices extracellulaires. Les complexes ne peuvent donc plus atteindre les cellules cibles, ce qui, par voie de

conséquence, entraîne une diminution de l'efficacité de transfert par rapport à la quantité de complexes injectée,

- et enfin, de nombreux acteurs du domaine de la transfection non-virale de gènes ont signalés que les lipides ou polymères cationiques ont un effet inflammatoire.

5           Par ailleurs, la formulation stable des vecteurs synthétique développés jusqu'à aujourd'hui à de faibles rapports de charges est en général difficile, voire impossible, et il a été constaté en outre qu'à faible rapport de charges, l'efficacité de transfert est souvent faible (Pitard et al., PNAS USA, 94, pp. 14412-14417, 1997). Dans toute la suite, "rapport de charge" signifie le rapport des charges positives de l'agent de  
10 transfert sur les charges négatives de l'ADN. Ce rapport est souvent exprimé en nmoles d'agent de transfert par  $\mu\text{g}$  d'ADN.

          Ce sont ces problèmes que les nouveaux agents transfectants mis au point par la demanderesse, et qui font l'objet de la présente invention, se proposent de résoudre. En effet, leur structure particulière forme une ancre hydrophobe liée d'une part à un  
15 polycation qui permet la formation de complexes avec les acides nucléiques et d'autre part à au moins une tête hydrophile qui permet de diminuer la densité de charge globale apparente de ces agents transfectants par rapport aux lipides ou aux polymères cationiques classiquement utilisés en transfection non-virale. La présence d'au moins une tête hydrophile crée une sorte d'« écran de charges » par diminution du potentiel  
20 zéta des complexes formés avec l'acide nucléique. Ainsi, lesdits complexes apparaissent moins cationiques à l'organisme, avec les conséquences bénéfiques qui en découlent. De plus, il a été montré que les agents transfectants selon la présente invention sont particulièrement avantageux d'un point de vue physico-chimique car ils sont particulièrement stables lorsqu'ils sont mis en contact avec des acides nucléiques  
25 à de faibles rapports de charge.

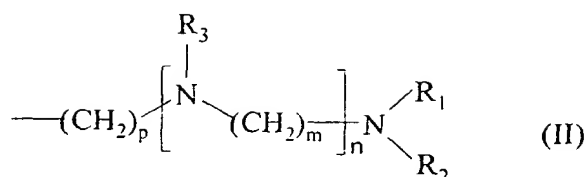
          Ainsi, un premier objet de l'invention concerne de nouveaux agents de transfert d'acides nucléiques qui comprennent un espaceur hydrophobe lié

chimiquement d'une part à un polycation et d'autre part à au moins un substituant hydrophile.

Le polycation permet de former des complexes avec les acides nucléiques par interactions avec les charges anioniques des acides nucléiques. L'espaceur hydrophobe a une double fonction. Il permet d'une part le passage à travers les membranes cellulaires, et d'autre part, il rend les complexes formés avec les acides nucléiques viables en milieu biologique. En effet, l'espaceur hydrophobe crée une contrainte physique sur les complexes qui permet de protéger les acides nucléiques du milieu extérieur. L'hydrophobicité nécessaire pour que les complexes soient viables peut être aisément déterminée par l'homme du métier, par application des méthodes de recherche ordinaires ou par la méthode habituelle des tâtonnements. Enfin, la présence du ou des substituants hydrophiles permet de diminuer le potentiel zéta des complexes formés, ce qui fait apparaître lesdits complexes moins cationiques au milieu extérieur.

Au sens de l'invention, le polycation est une molécule linéaire ou ramifiée polycationique susceptible de s'associer avec les acides nucléiques. On entend au sens de l'invention par association avec l'acide nucléique, tout type de liaisons comme par exemple les liaisons covalentes, les interactions électrostatiques, ioniques, les ponts hydrogène etc... De préférence, le polycation est une polyamine linéaire ou ramifiée, chaque groupe amino étant séparé par un ou plusieurs groupes méthylène. Eventuellement, la polyamine peut en outre être substituée par d'autres fonctions cationiques, par exemple des groupes amidinium ou guanidinium, des guanidines cycliques etc... Il peut s'agir notamment d'un polycation tel que défini dans les demandes de brevet WO 96/17823, WO 97/18185, WO 97/31935, WO 98/54130 ou encore WO 99/51581, et plus généralement dans toute la littérature concernant des structures de lipides cationiques connue de l'homme du métier. Selon un aspect préféré de l'invention, le polycation représente une polyamine de formule générale (II)

:



dans laquelle :

- $\text{R}_1$ ,  $\text{R}_2$  et  $\text{R}_3$  représentent indépendamment les uns des autres un atome d'hydrogène ou un groupement  $(\text{CH}_2)_q\text{NR}'\text{R}''$  avec  $q$  un nombre entier pouvant varier de 1 à 6, ceci de manière indépendante entre les différents groupements  $\text{R}_1$ ,  $\text{R}_2$  et  $\text{R}_3$ , étant entendu que l'un au moins de  $\text{R}_1$ ,  $\text{R}_2$  et  $\text{R}_3$  est différent d'un atome d'hydrogène,
- $\text{R}'$  et  $\text{R}''$  représentent indépendamment l'un de l'autre un atome d'hydrogène ou un groupement  $(\text{CH}_2)_q\text{NH}_2$  avec  $q$  défini comme précédemment,
- $m$  représente un nombre entier compris entre 1 et 6, et
- 10 -  $n$  et  $p$  représentent indépendamment l'un de l'autre des nombres entiers compris entre 0 et 6, avec lorsque  $n$  est supérieur ou égal à 2,  $m$  pouvant prendre des valeurs différentes et  $\text{R}_3$  des significations différentes au sein de la formule générale (II), et lorsque  $n$  est égal à 0, l'un au moins des substituants  $\text{R}_1$  et  $\text{R}_2$  est différent d'un atome d'hydrogène.
- 15 D'autres polycations possibles peuvent être également choisis parmi la spermine, la spermidine, la cadavérine, la putrescine, l'hexaméthylènetétramine (hexamine), le chlorure de méthacrylamidopropyl-triméthylammonium (AMBTAC), le chlorure de 3-acrylamido-3-méthylbutyltriméthylammonium (AMBTAC), les polyvinylamines, les polyéthylèneimines, ou encore les ionènes. (références : Barton et al.,
- 20 *Comprehensive Organic Chemistry*, Vol. 2, Ed. Pergamon Press, p. 90 ; *Encyclopedia of Polymer Science and Engineering*, 2<sup>nd</sup> Edition, Ed. Wiley Interscience, Vol. 11, p. 489 ; Mahler and Cordes, *Biological Chemistry*, Harper International Edition, p. 124.)

L'espaceur hydrophobe peut prendre des structures très variées du moment qu'il apporte une hydrophobicité suffisante pour permettre la protection des acides

25 nucléiques et le passage à travers les membranes. Cette hydrophobicité suffisante peut être déterminée par l'homme du métier en appliquant des méthodes de recherche

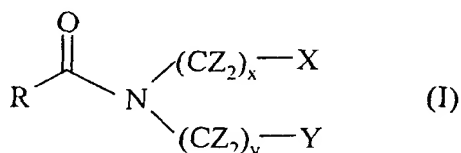
ordinaires. Selon une variante préférée de l'invention, l'espaceur hydrophobe est constitué de 2 ou 3 chaînes grasses linéaires hydrocarbonées (c'est-à-dire entre 10 et 20 atomes de carbone par chaîne, et de préférence 12, 14, 15, 16, 17, ou 18 atomes de carbone par chaîne, chaque chaîne pouvant être de longueur différente). Selon une  
5 autre variante, l'espaceur hydrophobe est constitué d'une très longue chaîne grasse linéaire hydrocarbonée, c'est-à-dire comprenant entre 20 et 50 atomes de carbone, et de préférence entre 40 et 50 atomes de carbone et encore plus préférentiellement entre 44 et 50 atomes de carbone).

Des substituants hydrophiles convenables sont par exemple choisis parmi les  
10 substituants hydroxy, amino, les polyols, les sucres, ou encore les peptides hydrophiles. On entend par polyol toute molécules hydrocarbonée linéaire, ramifiée ou cyclique comprenant au moins deux fonctions hydroxy. On peut citer à titre d'exemple le glycérol, l'éthylène glycol, le propylène glycol, les tétritols, les pentitols, les pentitols cycliques (ou quercitols), les hexitols comme le mannitol, le  
15 sorbitol, les dulcitol, les hexitols cycliques ou inositols etc...(Stanek et al., The Monosaccharidess Academic Press, pp. 621-655 et pp. 778-855).

Selon une variante avantageuse, les agents de transfert selon l'invention comprennent au moins un substituant hydrophile qui est un sucre. On entend au sens de l'invention par "sucre", toute molécule constituée d'un ou plusieurs saccharides.  
20 On peut citer à titre d'exemple des sucres tels que les pyranoses et les furanoses, par exemple le glucose, le mannose, le rhamnose, le galactose, le fructose, ou encore le maltose, le lactose, le saccharose, le sucrose, le fucose, le cellobiose, l'allose, le laminarabiose, le gentiobiose, le sophorose, le mélibiose etc... De préférence, le ou les sucres sont choisis parmi le glucose, le mannose, le rhamnose, le galactose, le  
25 fructose, le lactose, le saccharose et le cellobiose. De plus, il peut également s'agir de sucres dits "complexes", c'est-à-dire de plusieurs sucres couplés covalamment les uns aux autres, chaque sucre étant de préférence choisi dans la liste citée précédemment. A titre de polysaccharides convenables, on peut citer le dextran, l' $\alpha$ -amylose, l'amylopectine, les fructans, les mannans, les xylans et les arabinans. Certains sucres

préférés peuvent en outre interagir avec des récepteurs cellulaires, comme par exemple certains types de lectines.

Plus particulièrement, les agents de transfert selon l'invention peuvent être représentés par la formule générale (I) :



5

pour laquelle :

- R représente un polycation,
- Z représente un atome d'hydrogène ou un atome de fluor, les différents Z étant indépendant les uns des autres, et

10        - soit x et y, indépendamment l'un de l'autre, représentent des entiers compris entre 10 et 22 inclus, et X et Y, indépendamment l'un de l'autre, représentent un atome d'hydrogène, un groupement -OAlk où Alk représente un alkyle droit ou ramifié contenant 1 à 4 atomes de carbone, un groupe hydroxy, un groupement amino, un polyol, un sucre, un peptide hydrophile ou non-hydrophile, ou un oligonucléotide,

15        étant entendu que l'un au moins des substituants X et Y représente un groupe hydrophile choisi parmi les hydroxy, les amino, les polyols, les sucres, ou les peptides hydrophiles,

20        - soit x est égal à 0 ou 1, y est un entier compris entre 20 et 50, X est soit un atome d'hydrogène soit un groupement -OAlk où Alk représente un alkyle droit ou ramifié contenant 1 à 4 atomes de carbone, et Y est un groupe hydrophile choisi parmi les hydroxy, les amino, les polyols, les sucres, ou les peptides hydrophiles.

Au sens de l'invention, le polycation, les polyols et les sucres de la formule générale (I) sont tels que définis précédemment.

25        Les termes x et y sont définis dans la formule générale (I) de façon à prendre toute valeur comprise entre 10 et 22 inclus ou entre 20 et 50 inclus selon les cas. De

préférence, x et y, indépendamment l'un de l'autre, sont compris entre 12 et 18 inclus. Plus préférentiellement, x et y valent, indépendamment l'un de l'autre, 14, 15, 16, 17 ou 18. Lorsque x est égal à 0 ou 1, alors y est compris de préférence entre 30 et 50, ou entre 40 et 50. Plus préférentiellement, y est compris entre 44 et 50.

- 5           On entend au sens de l'invention par "oligonucléotide" des chaînes contenant un ou plusieurs nucléotides, désoxynucléotides, ribonucléotides et/ou désoxyribonucléotides qui sont des unités monomériques se différenciant les unes des autres par la présence de bases qui peuvent être choisies parmi l'adénine, la guanine, la cytosine, la thymidine ou l'uracile [voir Lehninger Biochimie, Flammarion
- 10   Medecine Sciences, 2nde édition, p. 305-329]. Du fait de leur propriété à former des paires de base, les oligonucléotides sont largement utilisés en biologie moléculaire, par exemple comme linkers (molécule de liaison) ou comme sondes.
- Par ailleurs, les oligonucléotides peuvent aussi être utilisés sous forme de conjugués, c'est-à-dire couplés à une ou plusieurs autres molécules ayant des propriétés
- 15   distinctes. A titre d'exemple, on peut citer le couplage d'un oligonucléotide avec un groupe chimique réactif, avec des groupes fluorescents ou chimioluminescents, ou encore avec des groupes susceptibles de favoriser les interactions intermoléculaires de façon à promouvoir l'entrée dans les cellules. De tels conjugués, décrits dans Bioconjugate Chemistry [John Goodchild, *Conjugates of Oligonucleotides and*
- 20   *Modified Oligonucleotides : a Review of their Synthesis and properties*, Vol. 1, N°3, 1990, pp. 165-187], possèdent de nombreuses utilisations et avantages comme par exemple la capacité d'améliorer l'entrée de complexes dans les cellules, de diminuer le taux de dégradation par les nucléases, d'augmenter la stabilité du complexe concerné, de suivre le devenir des oligonucléotides dans un organisme etc... Ainsi, le ou les
- 25   oligonucléotides, lorsqu'ils sont greffés sur les agents de transfert selon la présente invention, permettent d'apporter une propriété supplémentaire aux dits agents de transfert (par exemple des propriétés de ciblage, de marquage etc...).
- Les oligonucléotides peuvent être obtenus selon les méthodes classiques connues de l'homme du métier, et il est également possible de synthétiser des oligonucléotides



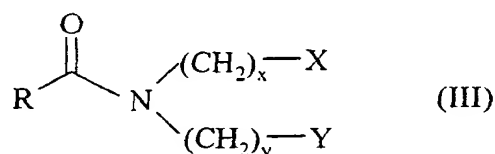
modifiés selon les méthodes décrites dans Bioconjugate Chemistry, John Goodchild, *Conjugates of Oligonucleotides and Modified Oligonucleotides : a Review of their Synthesis and properties*, Vol. 1, N°3, 1990, pp. 165-187 ou dans Tetrahedron, Beaucage et al., *The Synthesis of Modified Oligonucleotides by the Phosphoramidite Approach and Their Application*, Vol. 49, N° 28, pp. 6123-6194, 1993.

On entend au sens de l'invention par "peptide" des chaînes contenant un ou plusieurs acides aminés liés entre eux par des liaisons de nature peptidique [Lehninger Biochimie, Flammarion Medecine Sciences, 2nde édition]. Il peut s'agir des 20 acides aminés "classiques", c'est-à-dire ceux trouvés couramment dans la composition des protéines (Alamine, Valine, Leucine, Isoleucine, Proline, Phenylalamine, Tryptophane, Méthionine, Acide Aspartique, Glutamine, Lysine, Arginine, Histidine, Glycine, Sérine, Thréonine, Cystéine, Tyrosine, Asparagine, Acide Glutamique), ou bien il peut aussi s'agir des acides aminés dits "rares" comme par exemple la 4-hydroxyproline, la desmosine, la 5-hydroxylysine, la N-méthyllysine, la 3-méthylhistidine, l'isodesmosine etc... Enfin, il peut également s'agir des acides aminés apparaissant dans différentes cellules ou divers tissus sous forme libre ou combinée et qui dérivent en général des acides  $\alpha$ -aminés (par exemple la  $\beta$ -alamine, l'acide  $\gamma$ -aminobutyrique, l'homocystéine, l'ornithine, la canavanine, l'acide djenkalique, la  $\beta$ -cyanoalamine etc...). De tels peptides peuvent par exemple permettre le ciblage de certains types cellulaires. Dans ce contexte, on peut par exemple citer les peptides RGD ou NLS. Il peut également s'agir séquences peptidiques ayant des propriétés de marquage, c'est-à-dire permettant l'identification, par exemple par des techniques d'analyse comme la spectrométrie de fluorescence, la spectrométrie infrarouge, la résonance magnétique nucléaire (RMN) etc... On peut par exemple citer à ce titre les séquences peptidiques ou pseudopeptidiques linaires ou cycliques comportant l'épitope Arg-Gly-Asp (Arginine-Glycine-Acide Aspartique) de reconnaissance des récepteurs primaires et/ou secondaires des protéines d'adhésion du type intégrines. Les peptides selon l'invention peuvent en outre être substitués au niveau de un ou plusieurs de leurs groupes fonctionnels, par exemple au niveau du carboxyle en  $\alpha$ , de

- la fonction amine en  $\alpha$  et/ou au niveau des groupes fonctionnels de la chaîne latérale de chacun des acides aminés. A titre d'exemple, on peut citer les substitutions par des groupements aliphatiques saturés ou insaturés, linéaires, ramifiés ou cycliques contenant 1 à 24 atomes de carbone, tels que par exemple des radicaux cholestéryle, arachidonyle ou rétinoyle, ou encore des groupements mono- ou polyaromatiques tels que par exemple des dérivés benzyloxycarbonate, benzylester ou rhodaminyle substitués ou non. L'intérêt de telles substitutions s'inscrit dans le cadre de modification des propriétés chimiques et éventuellement biologiques desdits peptides, par exemple afin de les marquer.
- 10 Lorsque lesdits peptides sont utilisés à titre de substituant hydrophile, ceux-ci sont choisis parmi les peptides hydrophiles, c'est-à-dire les peptides constitués uniquement d'acides aminés hydrophiles ou encore ceux composés partiellement d'acides aminés hydrophiles et dont la composition les rend globalement hydrophiles.

Selon une variante préférée de l'invention, les groupes Z représentent tous des  
15 atomes d'hydrogène.

Selon un aspect plus particulièrement avantageux de l'invention, les agents de transfert sont de formule générale (III) :



pour laquelle :

- 20 - R représente un polycation, et  
- soit x et y, indépendamment l'un de l'autre, représentent des entiers compris entre 10 et 22 inclus, et X et Y, indépendamment l'un de l'autre, représentent un atome d'hydrogène ou un sucre, étant entendu que l'un au moins des substituants X et Y représente un sucre,
- 25 - soit x est égal à 0 ou 1, y est un entier compris entre 20 et 50, X est un atome d'hydrogène et Y est un sucre.

Au sens de l'invention, le polycation, les sucres et x et y dans la formule générale (III) sont tels que définis précédemment pour la formule générale (I).

Des agents de transfert plus particulièrement préférés sont de formule générale (III) et x et y, indépendamment l'un de l'autre, représentent des entiers compris entre 5 10 et 22 inclus, et l'un de X et Y représente un atome d'hydrogène et l'autre un sucre. Selon une autre variante avantageuse, les agents de transfert selon l'invention sont de formule générale (III) et x est égal à 0, y est un entier compris entre 40 et 50, X représente un atome d'hydrogène, et Y est un sucre.

Il est entendu que la présente invention concerne également les isomères des 10 produits de formule générale (I) lorsqu'ils existent, ainsi que leurs mélanges, ou leurs sels.

Notamment, les composés de l'invention peuvent se présenter sous forme de sels non toxiques et pharmaceutiquement acceptables. Ces sels non toxiques comprennent les sels avec les acides minéraux (par exemple l'acide chlorhydrique, 15 sulfurique, bromhydrique, phosphorique, nitrique), avec les acides organiques (acide acétique, propionique, succinique, maléique, hydroxymaléique, benzoïque, fumarique, méthanesulfonique ou oxalique), avec les bases minérales (soude, potasse, lithine, chaux), ou avec les bases organiques (amines tertiaires comme la triéthylamine, la pipéridine, la benzylamine).

20 Selon l'invention, la préparation des produits de formule générale (I) s'effectue en mettant en oeuvre les étapes suivantes :

1) On prépare dans un premier temps une chaîne alkyle à x atomes de carbone (x étant défini comme précédemment), comportant une fonction hydroxy et une fonction ester, par ouverture d'une lactone correspondante. La réaction s'effectue 25 généralement dans un alcool, à pH basique et à une température comprise entre -10°C et la température ambiante. A titre d'exemple, l'alcool peut être le méthanol ou l'éthanol.

- 2) Puis, on fixe le groupe X sur la chaîne alkyle bifonctionnelle obtenue à l'étape précédente. Lorsque X représente un sucre, on effectue une condensation dans un solvant chloré, comme par exemple le dichlorométhane ou le chloroforme, et en présence d'un acide de Lewis, à température comprise entre -5°C et 10°C. L'acide de Lewis peut par exemple être choisi parmi le chlorure d'étain, le chlorure de fer, l'acide p-toluène sulfonique (tsOH), le triméthylsilyltrifluorométhane sulfonique (TMStf), le trifluorure de bore étherate etc...[Kazunobu Toshima et al., *Recent Progress in O-glucosilation Methods and its Application to Natural Products Synthesis*, Chem. Rev. 1993, Vol. 93, pp. 1503-1531].
- 10 Lorsque X représente un groupe peptidique hydrophile ou non, on effectue un couplage peptidique selon les méthodes classiques (Bodanski M., *Principles and Practices of Peptides Synthesis*, Ed. Springe-Verlag) ou par toute méthode analogue connue de l'homme du métier. Notamment, la réaction s'effectue généralement en présence d'une base non-nucléophile dans des solvants aprotiques convenables, à
- 15 température comprise entre 0 et 100°C, le pH étant ajusté entre 9 et 11. A titre d'exemple, le chloroforme, la diméthylformamide, la méthylpyrrolidone, l'acétonitrile, le dichlorométhane, le toluène ou le benzène peuvent être utilisés comme solvant. Les bases non-nucléophiles employées sont préférentiellement des amines tertiaires, du carbonate de calcium ou du dicarbonate de sodium. Encore plus préférentiellement,
- 20 les bases utilisées sont des amines tertiaires comme par exemple la triéthylamine (TEA) ou le N-éthyl-diisopropylamine. Avantagusement, le couplage peptidique est effectué entre 0 et 50°C, et de préférence entre 10 et 30°C.
- Lorsque l'on souhaite que X représente un groupe hydroxy, cette étape n'est pas effectuée.
- 25 Lorsque X représente un groupe amino, la réaction est effectuée par substitution nucléophile selon les méthodes classiques connues de l'homme du métier qui permettent d'obtenir une amine à partir d'un alcool.
- Lorsque X représente un groupe -OAlk, on effectue une alkylation de la fonction alcool selon les méthodes classiques connues de l'homme du métier ou selon des
- 30 méthodes analogues. Par exemple, on peut faire réagir un composé diazo de formule

générale  $\text{Alk-N}_2$  éventuellement en présence d'un catalyseur tel que  $\text{HBF}_4$  ou du gel de silice. On peut également opérer dans les conditions de la Réaction de Williamson qui consiste à faire réagir en milieu basique un composé de formule générale  $\text{Alk-Hal}$  où Hal représente un atome d'halogène tel que le chlore, le brome ou l'iode, sur la

5 chaîne portant une fonction alcool.

La même réaction de type Williamson peut également être mise en œuvre lorsqu'on souhaite que X représente un polyol.

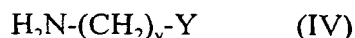
Enfin, lorsque X représente un oligonucléotide, celui-ci est couplé à la chaîne bifonctionnelle selon les méthodes classiques connues pour greffer covalamment un

10 oligonucléotide. Par exemple, ledit oligonucléotide peut être greffé par l'intermédiaire d'un linker (molécule de liaison) convenable.

3) Dans un troisième temps, la fonction ester présente sur la chaîne bifonctionnelle est hydrolysée en fonction acide selon les méthodes connues. Par exemple, on peut opérer en milieu basique dans un alcool à haut point d'ébullition, à

15 température comprise entre  $50^\circ\text{C}$  et la température de reflux du mélange réactionnel.

4) Puis, une chaîne alkylamine substituée ou non de formule générale (IV) :



dans laquelle y et Y sont définis comme précédemment, est couplée au composé obtenu à l'étape précédente, selon les méthodes de couplage peptidique classiques

20 (Bodanski M., *Principles and Practices of Peptides Synthesis*, Ed. Springer-Verlag) ou par toute méthode analogue connue de l'homme du métier.

Notamment, la réaction s'effectue généralement en présence d'une base non-nucléophile dans des solvants aprotiques convenables, à température comprise entre 0 et  $100^\circ\text{C}$ , le pH étant ajusté entre 9 et 11. A titre d'exemple, le chloroforme, la

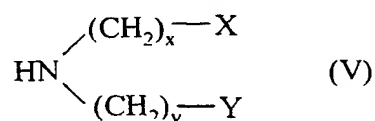
25 diméthylformamide, la méthylpyrrolidone, l'acétonitrile, le dichlorométhane, le toluène ou le benzène peuvent être utilisés comme solvant. Les bases non-nucléophiles employées sont préférentiellement des amines tertiaires, du carbonate de

calcium ou du dicarbonate de sodium. Encore plus préférentiellement, les bases utilisées sont des amines tertiaires comme par exemple la triéthylamine (TEA) ou le N-éthyl-diisopropylamine. Avantageusement, le couplage peptidique est effectué entre 0 et 50°C, et de préférence entre 10 et 30°C.

- 5 Le groupe de formule générale (IV) est soit disponible dans le commerce, soit il peut être obtenu par condensation de Y sur l'alkylamine non-substituée correspondante selon une méthode analogue à celle décrite précédemment en 2).

5) L'amide obtenue à l'étape précédente est ensuite réduite en amine. On opère pour cela selon les méthodes classiques connues de l'homme du métier. Par exemple, 10 on opère dans un solvant organique anhydre comme le tétrahydrofurane anhydre, par action d'hydrure de lithium aluminium  $\text{LiAlH}_4$ . D'autres agents réducteurs pouvant être utilisés sont par exemple le borane, le borane dans le diméthylsulfure ( $\text{BH}_3\text{-SMe}_2$ ), le borohydrure de sodium/tétrachlorure de titane ( $\text{NaBH}_4, \text{TiCl}_4$ ), le chlorure d'oxyde de phosphore sur Zinc ( $\text{POCl}_3/\text{Zn}$ ), le pentasulfure de phosphore ( $\text{P}_4\text{S}_{10}$ ) sur 15 nickel de Raney, etc... [Richard C. Larock, *Comprehensive Organic Transformations*, VCH Publishers Inc., 1989]. On peut également opérer par hydrogénation catalytique. Avantageusement, la réduction est effectuée par action d'hydrure de lithium aluminium  $\text{LiAlH}_4$ , dans du tétrahydrofurane anhydre, à température de reflux du mélange.

- 20 On obtient ainsi un composé de formule générale (V) :



pour laquelle X, Y, x et y sont définis comme précédemment.

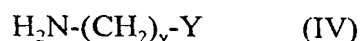
- 6) Enfin, dans une dernière étape, le dérivé acide correspondant au polycation R tel que défini précédemment, est couplé au composé de formule générale (IV) 25 obtenu à l'étape précédente, selon les méthodes de couplage peptidique classiques (Bodanski M., *Principles and Practices of Peptides Synthesis*, Ed. Springer-Verlag) ou par toute méthode analogue connue de l'homme du métier.

Notamment, la réaction s'effectue généralement en présence d'une base non-nucléophile dans des solvants aprotiques convenables, à température comprise entre 0 et 100°C, le pH étant ajusté entre 9 et 11. A titre d'exemple, le chloroforme, la diméthylformamide, la méthylpyrrolidone, l'acétonitrile, le dichlorométhane, le toluène ou le benzène peuvent être utilisés comme solvant. Les bases non-nucléophiles employées sont préférentiellement des amines tertiaires, du carbonate de calcium ou du dicarbonate de sodium. Encore plus préférentiellement, les bases utilisées sont des amines tertiaires comme par exemple la triéthylamine (TEA) ou le N-éthyl-diisopropylamine. Avantageusement, le couplage peptidique est effectué entre 0 et 50°C, et de préférence entre 10 et 30°C.

Les dérivés acides correspondant au polycation sont disponibles commercialement.

Selon une autre variante, les agents transfectants selon la présente invention peuvent être préparés en opérant de la façon suivante :

- 1) On prépare dans un premier temps une chaîne alkyle à x atomes de carbone (x étant défini comme précédemment), comportant une fonction hydroxy et une fonction ester, par ouverture d'une lactone correspondante. La réaction s'effectue généralement dans un alcool, à pH basique et à une température comprise entre -10°C et la température ambiante. A titre d'exemple, l'alcool peut être le méthanol ou l'éthanol.
- 2) Puis, on effectue sur cette chaîne alkyle bifonctionnelle le couplage d'une chaîne alkylamine substituée ou non de formule générale (IV) :



dans laquelle y et Y sont définis comme précédemment dans la formule générale (I). La réaction s'effectue à une température supérieure au point de fusion de chaque produit, avec ou sans vide. La réaction peut également être mise en œuvre à la température de reflux en présence d'un solvant alcoolique. A titre d'exemple, le

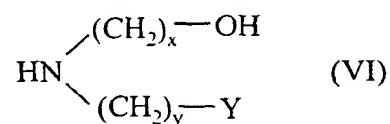
solvant peut être le méthanol ou l'éthanol. On opère par exemple à température comprise entre 45°C et 60°C.

La réaction peut également être menée à température de reflux du mélange en présence d'un alcool tel que le méthanol à titre de solvant. Une autre alternative  
5 consiste à effectuer le couplage du composé de formule générale (IV) directement avec la lactone (dans ce cas, la première étape d'ouverture de la lactone n'est pas effectuée).

Le groupe de formule générale (IV) est soit disponible dans le commerce, soit il peut être obtenu par condensation de Y sur l'alkylamine non-substituée correspondante  
10 selon une méthode analogue à celle décrite ci-avant.

3) l'amide bicaténaire bifonctionnelle obtenue est ensuite réduite en amine. On opère pour cela selon des méthodes classiques. Par exemple, on opère dans un solvant organique anhydre comme le tétrahydrofurane anhydre, par action d'hydruire de lithium et d'aluminium ( $\text{LiAlH}_4$ ). D'autres agents réducteurs qui peuvent être utilisés  
15 sont par exemple le borane, le diméthyle de sulfure d'hydruire de bore ( $\text{BH}_3\text{-SMe}_2$ ), le borohydruire de sodium/tétrachlorure de titane ( $\text{NaBH}_4$ ,  $\text{TiCl}_4$ ), le chlorure d'oxyde de phosphore sur Zinc ( $\text{POCl}_3/\text{Zn}$ ), le pentasulfure de phosphore ( $\text{P}_4\text{S}_{10}$ ) sur nickel de Raney, etc... [Richard C. Larock, Comprehensive Organic Transformations, VCH Publishers Inc., 1989]. On peut également opérer par hydrogénation catalytique.  
20 Avantagusement, la réduction est effectuée par action d'hydruire de lithium aluminium  $\text{LiAlH}_4$ , dans du tétrahydrofurane anhydre, à température de reflux du mélange.

On obtient ainsi un composé de formule générale (VI) :



25 pour laquelle Y, x et y sont définis comme précédemment.



4) Puis, on condense le groupe X sur l'amine de formule générale (VI) obtenue à l'étape précédente. La condensation est effectuée selon des méthodes analogues à celles décrites précédemment pour la première voie de synthèse.

- 5) Enfin, dans une dernière étape, le dérivé acide correspondant au polycation  
5 R tel que défini précédemment, est couplé au composé de formule générale (VI) obtenu à l'étape précédente, selon les méthodes de couplage peptidique classiques (Bodanski M., *Principles and Practices of Peptides Synthesis*, Ed. Sprunge-Verlag) ou par toute méthode analogue connue de l'homme du métier.

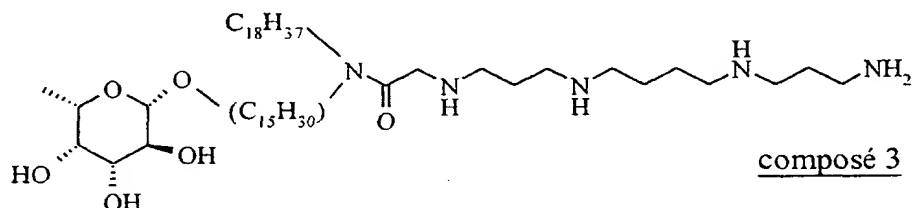
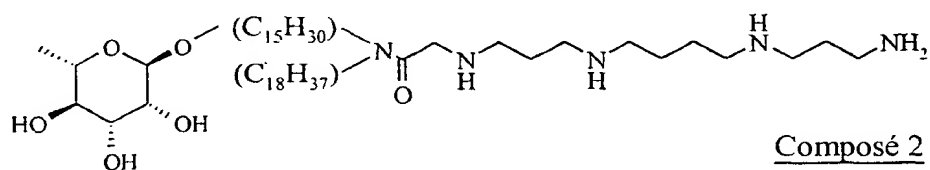
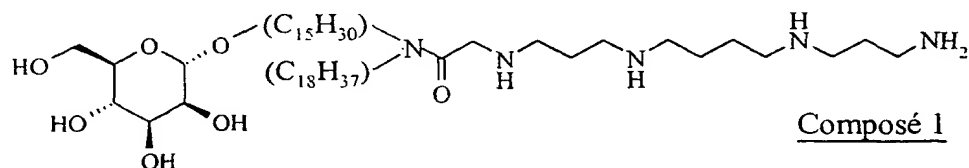
Notamment, la réaction s'effectue généralement en présence d'une base non-  
10 nucléophile dans des solvants aprotiques convenables, à température comprise entre 0 et 100°C, le pH étant ajusté entre 9 et 11. A titre d'exemple, le chloroforme, la diméthylformamide, la méthylpyrrolidone, l'acétonitrile, le dichlorométhane, le toluène ou le benzène peuvent être utilisés comme solvant. Les bases non-nucléophiles employées sont préférentiellement des amines tertiaires, du carbonate de  
15 calcium ou du dicarbonate de sodium. Encore plus préférentiellement, les bases utilisées sont des amines tertiaires comme par exemple la triéthylamine (TEA) ou le N-éthyl-diisopropylamine. Avantagusement, le couplage peptidique est effectué entre 0 et 50°C, et de préférence entre 10 et 30°C.

Les dérivés acides correspondant au polycation sont disponibles commercialement.

- 20 Naturellement, lorsque les substituants de X, Y et/ou du polycation peuvent interférer avec la réaction, il est préférable de les protéger préalablement avec des radicaux compatibles et pouvant être mis en place et éliminés sans toucher au reste de la molécule. On opère pour cela selon les méthodes classiques connues de l'homme du métier, et notamment selon les méthodes décrites dans T.W. GREENE, *Protective*  
25 *Groups in Organic Synthesis*, 2nd Edition, Wiley-Interscience, dans McOMIE, *Protective Groups in Organic Chemistry*, Plenum Press (1973), ou dans Philip J Kocienski, *Protecting Groups*, Thieme.

Par ailleurs, chaque étape du procédé de préparation peut être suivie, le cas échéant, des étapes de séparation et de purification du composé obtenu selon les méthodes connues de l'homme du métier.

A titre d'exemple illustratif d'agents de transfert d'acides nucléiques  
5 avantageux selon l'invention, on peut citer les composés suivants :



Un autre objet de l'invention concerne les compositions comprenant un agent  
10 de transfert d'acides nucléiques tel que défini ci-avant, et un acide nucléique. Les quantités respectives de chaque composant peut être ajusté aisément par l'homme du métier en fonction de l'agent de transfert utilisé, de l'acide nucléique, et des applications recherchées (notamment du type de cellules à transfecter).

On entend au sens de l'invention par "acide nucléique" aussi bien un acide  
15 désoxyribonucléique qu'un acide ribonucléique. Il peut s'agir de séquences naturelles ou artificielles, et notamment d'ADN génomique (ADNg), d'ADN complémentaire (ADNc), d'ARN messager (ARNm), d'ARN de transfert (ARNt), d'ARN ribosomique (ARNr), de séquences hybrides ou de séquences synthétiques ou semi-synthétiques,

d'oligonucléotides modifiés ou non. Ces acides nucléiques peuvent être d'origine humaine, animale, végétale, bactérienne, virale, etc... Ils peuvent être obtenus par toute technique connue de l'homme du métier, et notamment par criblage de banques, par synthèse chimique, ou encore par des méthodes mixtes incluant la modification chimique ou enzymatique de séquences obtenues par criblage de banques. Ils peuvent être modifiés chimiquement.

Concernant plus particulièrement les acides désoxyribonucléiques, ils peuvent être simple ou double brin de même que des oligonuléotides courts ou des séquences plus longues. En particulier, les acides nucléiques sont avantageusement constitués par des plasmides, des vecteurs, des épisomes, des cassettes d'expression, etc... Ces acides désoxyribonucléiques peuvent porter une origine de réplication fonctionnelle ou non dans la cellule cible, un ou plusieurs gènes marqueurs, des séquences régulatrices de la transcription ou de la réplication, des gènes d'intérêt thérapeutique, des séquences antisens modifiées ou non, des régions de liaison à d'autres composants cellulaires, etc...

De préférence, l'acide nucléique comprend un ou plusieurs gènes d'intérêt thérapeutique sous contrôle de séquences de régulation, par exemple un ou plusieurs promoteurs et un terminateur transcriptionnel actifs dans les cellules cibles.

Au sens de l'invention, on entend par gène d'intérêt thérapeutique notamment tout gène codant pour un produit protéique ayant un effet thérapeutique. Le produit protéique ainsi codé peut être notamment une protéine ou un peptide. Ce produit protéique peut être exogène homologue ou endogène vis-à-vis de la cellule cible, c'est-à-dire un produit qui est normalement exprimé dans la cellule cible lorsque celle-ci ne présente aucune pathologie. Dans ce cas, l'expression d'une protéine permet par exemple de pallier une expression insuffisante dans la cellule ou l'expression d'une protéine inactive ou faiblement active en raison d'une modification, ou encore de surexprimer ladite protéine. Le gène d'intérêt thérapeutique peut aussi coder pour un mutant d'une protéine cellulaire, ayant une stabilité accrue, une activité

modifiée, etc... Le produit protéique peut également être hétérologue vis-à-vis de la cellule cible. Dans ce cas, une protéine exprimée peut par exemple compléter ou apporter une activité déficiente dans la cellule, lui permettant de lutter contre une pathologie, ou stimuler une réponse immunitaire.

5            Parmi les produits thérapeutiques au sens de la présente invention, on peut citer plus particulièrement les enzymes, les dérivés sanguins, les hormones, les lymphokines : interleukines, interférons, TNF, etc... (FR 92/03120), les facteurs de croissance, les neurotransmetteurs ou leurs précurseurs ou enzymes de synthèse, les facteurs trophiques (BDNF, CNTF, NGF, IGF, GMF, aFGF, bFGF, NT3, NT5,  
10 HARP/pléiotrophine, etc...) les apolipoprotéines (ApoAI, ApoAIV, ApoE, etc..., FR 93/05125), la dystrophine ou une minidystrophine (FR 91/11947), la protéine CFTR associée à la mucoviscidose, les gènes suppresseurs de tumeurs (p53, Rb, Rap1A, DCC, k-rev, etc..., FR 93/04745), les gènes codant pour des facteurs impliqués dans  
15 l'ADN, les gènes suicides (thymidine kinase, cytosine déaminase), les gènes de l'hémoglobine ou d'autres transporteurs protéiques, les enzymes du métabolisme, catabolisme etc...

            L'acide nucléique d'intérêt thérapeutique peut également être un gène ou une séquence antisens, dont l'expression dans la cellule cible permet de contrôler  
20 l'expression de gènes ou la transcription d'ARNm cellulaires. De telles séquences peuvent, par exemple, être transcrites dans la cellule cible en ARN complémentaires d'ARNm cellulaires et bloquer ainsi leur traduction en protéine, selon la technique décrite dans le brevet EP 140 308. Les gènes thérapeutiques comprennent également  
25 les séquences codant pour des ribozymes, qui sont capables de détruire sélectivement des ARN cibles (EP 321 201).

            Comme indiqué plus haut, l'acide nucléique peut également comporter un ou plusieurs gènes codant pour un peptide antigénique, capable de générer chez l'homme ou l'animal une réponse immunitaire. Dans ce mode particulier de mise en oeuvre,

l'invention permet la réalisation soit de vaccins soit de traitements immunothérapeutiques appliqués à l'homme ou à l'animal, notamment contre des micro-organismes, des virus ou des cancers. Il peut s'agir notamment de peptides antigéniques spécifiques du virus d'Epstein Barr, du virus HIV, du virus de l'hépatite B (EP 185 573), du virus de la pseudo-rage, du "syncytia forming virus", d'autres virus ou encore de peptides antigéniques spécifiques de tumeurs (EP 259 212).

Préférentiellement, l'acide nucléique comprend également des séquences permettant l'expression du gène d'intérêt thérapeutique et/ou du gène codant pour le peptide antigénique dans la cellule ou l'organe désiré. Il peut s'agir des séquences qui sont naturellement responsables de l'expression du gène considéré lorsque ces séquences sont susceptibles de fonctionner dans la cellule infectée. Il peut également s'agir de séquences d'origine différente (responsables de l'expression d'autres protéines, ou même synthétiques). Notamment, il peut s'agir de séquences promotrices de gènes eucaryotes ou viraux. Par exemple, il peut s'agir de séquences promotrices issues du génome de la cellule que l'on désire infecter. De même, il peut s'agir de séquences promotrices issues du génome d'un virus. A cet égard, on peut citer par exemple les promoteurs des gènes E1A, MLP, CMV, RSV, etc... En outre, ces séquences d'expression peuvent être modifiées par addition de séquences d'activation, de régulation, etc... Il peut aussi s'agir de promoteur, inductible ou répressible.

Par ailleurs, l'acide nucléique peut également comporter, en particulier en amont du gène d'intérêt thérapeutique, une séquence signal dirigeant le produit thérapeutique synthétisé dans les voies de sécrétion de la cellule cible. Cette séquence signal peut être la séquence signal naturelle du produit thérapeutique, mais il peut également s'agir de toute autre séquence signal fonctionnelle, ou d'une séquence signal artificielle. L'acide nucléique peut également comporter une séquence signal dirigeant le produit thérapeutique synthétisé vers un compartiment particulier de la cellule.

Les compositions selon l'invention peuvent en outre comporter un ou plusieurs adjuvants capables de s'associer aux complexes agent de transfert/acide nucléique et d'en améliorer le pouvoir transfectant. Dans un autre mode de mise en oeuvre, la présente invention concerne donc des compositions comprenant un acide nucléique, un agent de transfert d'acides nucléiques tel que défini ci-avant et au moins un adjuvant capable de s'associer aux complexes agent de transfert/acide nucléique et d'en améliorer le pouvoir transfectant. La présence de ce type d'adjuvant (lipides, peptides ou protéines par exemple) peut permettre avantageusement d'augmenter le pouvoir transfectant des composés. Dans cette optique, les compositions de l'invention peuvent comprendre à titre d'adjuvant, un ou plusieurs lipides neutres.

Plus préférentiellement, les lipides neutres utilisés dans le cadre de la présente invention sont des lipides à deux chaînes grasses. De manière particulièrement avantageuse, on utilise des lipides naturels ou synthétiques, zwitterioniques ou dépourvus de charge ionique dans les conditions physiologiques. Ils peuvent être choisis plus particulièrement parmi la dioléoylphosphatidyléthanolamine (DOPE), l'oléoylpalmitoylphosphatidyléthanolamine (POPE), les distéaroyl-, -palmitoyl-, -mirystoylphosphatidyléthanolamines ainsi que leurs dérivés N-méthylés 1 à 3 fois, les phosphatidylglycérols, les diacylglycérols, les glycosyldiacylglycérols, les cébrosides (tels que notamment les galactocébrosides), les sphingolipides (tels que notamment les sphingomyélines) ou encore les asialogangliosides (tels que notamment les asialoGM1 et GM2).

Ces différents lipides peuvent être obtenus soit par synthèse, soit par extraction à partir d'organes (exemple : le cerveau) ou d'oeufs, par des techniques classiques bien connues de l'homme du métier. En particulier, l'extraction des lipides naturels peut être réalisée au moyen de solvants organiques (voir également Lehninger, Biochemistry).

Plus récemment, la demanderesse a démontré qu'il était également particulièrement avantageux d'employer à titre d'adjuvant, un composé intervenant

ou non directement au niveau de la condensation dudit acide nucléique, tels que ceux décrits dans la demande de brevet WO 96/25508. La présence d'un tel composé, au sein d'une composition selon l'invention, permet de diminuer la quantité d'agent transfectant, avec les conséquences bénéfiques qui en découlent sur le plan toxicologique, sans porter un préjudice quelconque à l'activité transfectante. Par composé intervenant au niveau de la condensation de l'acide nucléique, on entend définir un composé compactant, directement ou non, l'acide nucléique. Plus précisément, ce composé peut soit agir directement au niveau de l'acide nucléique à transfecter soit intervenir au niveau d'un composé annexe qui lui est directement impliqué dans la condensation de cet acide nucléique. De préférence, il agit directement au niveau de l'acide nucléique. Notamment, l'agent précompactant peut être tout polycation, par exemple la polylysine. Selon un mode de réalisation préféré, l'agent intervenant au niveau de la condensation de l'acide nucléique dérive en tout ou partie d'une protamine, d'une histone, ou d'une nucléoline et/ou de l'un de leurs dérivés. Un tel agent peut également être constitué, en tout ou partie, de motifs peptidiques (KTPKKAKKP) et/ou (ATPAKKAA), le nombre des motifs pouvant varier entre 2 et 10. Dans la structure du composé selon l'invention, ces motifs peuvent être répétés de manière continue ou non. C'est ainsi qu'ils peuvent être séparés par des liens de nature biochimique, par exemple par un ou plusieurs acides aminés, ou de nature chimique.

Préférentiellement, les compositions de l'invention comprennent de 0,01 à 20 équivalents d'adjuvant pour un équivalent d'acide nucléique en mol/mol et, plus préférentiellement, de 0,5 à 5.

Dans un mode de réalisation particulièrement avantageux, les compositions selon la présente invention comprennent en outre un élément de ciblage permettant d'orienter le transfert de l'acide nucléique. Cet élément de ciblage peut être un élément de ciblage extracellulaire permettant d'orienter le transfert de l'ADN vers certains, types cellulaires ou certains tissus souhaités (cellules tumorales, cellules hépatiques, cellules hématopoïétiques...). Il peut également s'agir d'un élément de ciblage

intracellulaire permettant d'orienter le transfert de l'acide nucléique vers certains compartiments cellulaires privilégiés (mitochondries, noyau etc...). L'élément de ciblage peut être lié à l'agent de transfert d'acides nucléiques selon l'invention, ou également à l'acide nucléique comme cela a été précisé précédemment. Lorsque  
5 l'élément de ciblage est lié à l'agent de transfert d'acides nucléiques de formule générale (I), celui-ci constitue de préférence l'un des substituants X ou Y.

Parmi les éléments de ciblage utilisables dans le cadre de l'invention, on peut citer les sucres, les peptides, les protéines, les oligonucléotides, les lipides, les neuromédiateurs, les hormones, les vitamines ou leurs dérivés. Préférentiellement, il  
10 s'agit de sucres, de peptides ou de protéines tels que des anticorps ou des fragments d'anticorps, des ligands de récepteurs cellulaires ou des fragments de ceux-ci, des récepteurs ou des fragments de récepteurs, etc... En particulier, il peut s'agir de ligands de récepteurs de facteurs de croissance, de récepteurs de cytokines, de récepteurs de type lectines cellulaires, ou de ligands à séquence RGD avec une  
15 affinité pour les récepteurs de protéines d'adhésion comme les intégrines. On peut également citer les récepteurs de la transferrine, des HDL et des LDL, ou le transporteur du folate. L'élément de ciblage peut également être un sucre permettant de cibler des lectines tels que les récepteurs aux asialoglycoprotéines ou aux sialydes tel que le sialyl Lewis X, ou encore un fragment Fab d'anticorps, ou un anticorps  
20 simple chaîne (ScFv).

L'association des éléments de ciblage aux complexes nucléolipidiques peut être effectuée par toute technique connue de l'homme du métier, par exemple par couplage à une partie hydrophobe ou à une partie qui interagit avec l'acide nucléique de l'agent de transfert selon l'invention, ou encore à un groupement qui interagit avec  
25 l'agent de transfert selon l'invention ou avec l'acide nucléique. Les interactions en question peuvent être, selon un mode préféré, de nature ionique ou covalente.

L'invention a également pour objet l'utilisation des composés tels que définis ci-avant pour le transfert de polynucléotides (et plus généralement de polyanions)



dans les cellules *in vitro*, *in vivo* ou *ex vivo*. Plus précisément, la présente invention a pour objet l'utilisation des composés tels que définis ci-avant pour la préparation d'un médicament destiné à traiter les maladies, en particulier les maladies qui résultent d'une déficience en un produit protéique ou nucléique. Le polynucléotide contenu

5 dans ledit médicament code ledit produit protéique ou nucléique, ou constitue ledit produit nucléique, apte à corriger lesdites maladies *in vivo* ou *ex vivo*.

Pour des utilisations *in vivo*, par exemple en thérapie ou pour l'étude de la régulation de gènes ou la création de modèles animaux de pathologies, les compositions selon l'invention peuvent être formulées en vue d'administrations par

10 voie topique, cutanée, orale, rectale, vaginale, parentérale, intranasale, intraveineuse, intramusculaire, sous-cutanée, intraoculaire, transdermique, intratrachéale, intrapéritonéale, etc... De préférence, les compositions de l'invention contiennent un véhicule pharmaceutiquement acceptable pour une formulation injectable, notamment pour une injection directe au niveau de l'organe désiré, ou pour une administration par

15 voie topique (sur peau et/ou muqueuse). Il peut s'agir en particulier de solutions stériles, isotoniques, ou de compositions sèches, notamment lyophilisées, qui, par addition selon le cas d'eau stérilisée ou de sérum physiologique, permettent la constitution de solutés injectables. Les doses d'acides nucléiques utilisées pour l'injection ainsi que le nombre d'administrations peuvent être adaptées en fonction de

20 différents paramètres, et notamment en fonction du mode d'administration utilisé, de la pathologie concernée, du gène à exprimer, ou encore de la durée du traitement recherchée. En ce qui concerne plus particulièrement le mode d'administration, il peut s'agir soit d'une injection directe dans les tissus, par exemple au niveau des tumeurs, ou dans les voies circulatoires, soit d'un traitement de cellules en culture suivi de leur

25 réimplantation *in vivo*, par injection ou greffe. Les tissus concernés dans le cadre de la présente invention sont par exemple les muscles, la peau, le cerveau, les poumons, le foie, la rate, la moelle osseuse, le thymus, le coeur, la lymphe, le sang, les os, les cartilages, le pancréas, les reins, la vessie, l'estomac, les intestins, les testicules, les

ovaires, le rectum, le système nerveux, les yeux, les glandes, les tissus conjonctifs, etc...

Un autre objet de la présente invention concerne une méthode de traitement du corps humain ou animal comprenant les étapes suivantes :

- 5 (1) la mise en contact de l'acide nucléique avec un agent de transfert tel que défini ci-avant, pour former un complexe, et  
(2) la mise en contact des cellules du corps humain ou animal avec le complexe formé en (1).

L'invention concerne en outre une méthode de transfert d'acides nucléiques  
10 dans les cellules comprenant les étapes suivantes :

- (1) la mise en contact de l'acide nucléique avec un agent de transfert tel que défini ci-avant, pour former un complexe, et  
(2) la mise en contact des cellules avec le complexe formé en (1).

La mise en contact des cellules avec le complexe peut être réalisée par  
15 incubation des cellules avec ledit complexe (pour des utilisations *in vitro* ou *ex vivo*), ou par injection du complexe dans un organisme (pour des utilisations *in vivo*). L'incubation est réalisée de préférence en présence de par exemple de 0,01 à 1000  $\mu\text{g}$  d'acide nucléique pour  $10^6$  cellules. Pour une administration *in vivo*, des doses d'acide nucléique allant de 0,01 à 10 mg peuvent par exemple être utilisées.

20 Dans le cas où les compositions de l'invention contiennent en outre un ou plusieurs adjuvants tels que définis précédemment, le ou les adjuvants sont préalablement mélangés à l'agent de transfert selon l'invention et/ou à l'acide nucléique.

La présente invention fournit ainsi une méthode particulièrement  
25 avantageuse pour le transfert d'acides nucléiques *in vivo*, notamment pour le traitement de maladies, comprenant l'administration *in vivo* ou *in vitro* d'un acide nucléique codant pour une protéine ou pouvant être transcrit en un acide nucléique

apte à corriger ladite maladie, ledit acide nucléique étant associé à un composé de formule générale (I) dans les conditions définies ci-avant.

Les agents de transfert d'acides nucléiques de l'invention sont particulièrement utiles pour le transfert d'acides nucléiques dans des cellules primaires ou dans des lignées établies. Il peut s'agir de cellules fibroblastiques, musculaires, nerveuses (neurones, astrocytes, cellules gliales), hépatiques, hématopoïétiques (lymphocytes, CD34, dendritiques, etc...), épithéliales etc..., sous forme différenciées ou pluripotentes (précurseurs).

Outre les dispositions qui précèdent, la présente invention comprend également d'autres caractéristiques et avantages qui ressortiront des exemples et figures qui suivent, et qui doivent être considérés comme illustrant l'invention sans en limiter la portée. Notamment, la demanderesse propose à titre non-limitatif divers protocoles opératoires ainsi que des intermédiaires réactionnels susceptibles d'être mis en oeuvre pour préparer les agents de transfert de formule générale (I). Bien entendu, il est à la portée de l'homme du métier de s'inspirer de ces protocoles ou produits intermédiaires pour mettre au point des procédés analogues en vue de conduire à ces mêmes composés. Il appartient également à l'homme du métier de s'inspirer des procédés de synthèse décrits dans les différentes demandes de brevet précédemment citées pour la synthèse du polycation R compris dans la formule générale (I) (WO 96/17823, WO 97/18185, WO 97/31935 etc...).

## **FIGURES**

Figure 1 : Représentation schématique du plasmide pXL2774 utilisé dans les expériences de transfert d'ADN dans les cellules.

Figure 2 : Activité de transfert de gène *in vitro* dans des cellules HeLa des complexes formés à partir du composé 2 selon l'invention sans co-lipide, ou bien en présence de cholestérol et en présence de DOPE comme co-lipides. L'axe des ordonnées représente

l'expression de la luciférase en pg/puit. L'axe des abscisses indique le rapport agent transfectant/ADN en nmole/ $\mu$ g d'ADN.

Figure 3 : Activité de transfert de gène *in vivo* après injection directe dans le muscle antérieur du tibia de souris de complexes formés à partir du composé 2 selon la présente invention en présence de DOPE (1 : 1). L'axe des ordonnées indique l'expression de la luciférase en pg/muscle. L'axe des abscisses indique le rapport composé 2/ADN en nmoles/ $\mu$ g d'ADN.

## EXEMPLES

### A \ MATÉRIEL ET MÉTHODES

#### 10 a) Matériel

- Les polyamines de départ, comme la spermidine, la spermine, le tris-(2-aminoéthyle)amine, la phénylènediamine, les diaminoalcane etc..., sont disponibles commercialement ou elles peuvent être synthétisée par des méthodes classiques (par exemple par cyanoéthylation d'amines disponibles dans le commerce pour obtenir des amines branchées)
- de nombreux composés comme par exemple la triéthylamine, l'hexafluorophosphate de benzotriazol-1-yloxytris(diméthylamino)phosphonium (BOP), le chloroformate de benzyle, le 11-bromoundécanol, etc... sont également des produits commerciaux.
- L'amberlite IR 120 est une résine échangeuse d'ions commerciale (catalogue BDH).
- 20 • Le diméthylsulfoxyde (DMSO), traité préalablement à l'hydroxyde de potassium, a été distillé sur hydrure de calcium puis stocké sur tamis moléculaire 4 Å.
- Le dichlorométhane a été distillé sur le pentoxyde de phosphore puis stocké sur tamis moléculaire 4 Å.
- Le tétrahydrofurane (THF) a été distillé sur sodium en présence de benzophénone.
- 25 • Pour les réactions nécessitant des conditions anhydre, toute la verrerie est séchée à la flamme sous courant d'azote.

#### b) Méthodes

- Analyses spectroscopiques

Les spectres de résonance magnétique nucléaire (RMN) ont été enregistrés sur un spectromètre Bruker MSL 30 à la fréquence de 300 MHz pour le proton et 75 MHz pour le carbone. Tous les déplacements chimiques sont reportés en ppm soit par rapport à la fréquence du tétraméthylsilane (TMS), soit par rapport au solvant. Les spectres ont été enregistrés en utilisant soit le TMS soit le signal résiduel du solvant comme référence interne. La multiplicité des signaux est désignée par les abréviations suivantes : s (singulet), d (doublet), t (triplet), q (quadruplet) et m (multiplet).

- Techniques de chromatographie

- 10 • La cinétique des réactions a été suivie par chromatographie sur couche mince (CCM) avec un gel de silice contenant un indicateur fluorescent (Merck Silicagel 60 F254) comme support. Les chromatogrammes ont été révélés par pulvérisation d'une solution alcoolique d'anisaldéhyde.
- 15 • Toutes les chromatographies sur colonne ont été réalisées sous pression d'air comprimé avec du silicagel 60 comme phase stationnaire (0,05-0,02 mm). La phase mobile employée diffère selon le type de synthèse (chromatographie moyenne pression).
- 20 • Les analyses CLHP (Chromatographie Liquide Haute Performance) sont réalisées sur un appareil Waters LC 4000 équipé d'une colonne analytique de type C4 commercialisée par Applied Biosystem ("Brownlee Columns" en acier inoxydable de 3 cm de longueur et 0,46 cm de diamètre) et d'un détecteur "Waters 486" à 220 nm. La phase stationnaire est de l'aquapore butyl 7 microns, et les phases mobiles sont l'eau déminéralisée (2500 cm<sup>3</sup>) ou l'acétonitrile (2500 cm<sup>3</sup>) additionné d'acide trifluoroacétique (2,5 cm<sup>3</sup>). Le débit est de 1 ml par minute.

25 **B \ SYNTHÈSE DES AGENTS DE TRANSFECTION**

Exemple 1 : synthèse de l' $\alpha$ -D-mannopyrannoside de (3-[4-(3-amino-propyl-amino)-butyl-amino]-méthylène-carbamoyl)-15-pentadécanyl-16-octadécyle  
(Composé 1)

5 *a) Synthèse de l'acide 3-[4-(3-tert-butoxycarbonyl-amino-propyl-tert-butoxycarbonyl-amino)-butyl-tert-butoxycarbonyl-amino] acétique (FRM 375)*

A une solution de spermine (5 g ; 24,96 mmoles) dans du méthanol (125 ml), on ajoute du cyanoborohydrure de sodium  $\text{NaBH}_3\text{CN}$  (0,548 g ; 8,74 mmoles). La solution est ensuite soumise à une vive agitation. Par l'intermédiaire d'une ampoule isobare, on ajoute en 100 minutes une solution d'acide glyoxylique (2,34 g ; 25,46  
10 mmoles) dans du méthanol (80 ml). Après une nuit, on ajoute au mélange de la triéthylamine (3,86 ml ; 27,71 mmoles) et du di-tert-butyl dicarbonate (27,67 g ; 129,79 mmoles) solubilisé dans du tétrahydrofurane (55 ml). Après une nuit, on concentre à l'évaporateur rotatif puis on reprend par de l'acétate d'éthyle (63 ml) et on effectue un lavage à l'hydrogénosulfate de potassium et à la saumure. Puis on sèche  
15 sur sulfate de magnésium et on concentre. Le produit obtenu est purifiée par chromatographie ( $\text{CH}_2\text{Cl}_2/\text{MeOH}$  9 : 1). Le rendement est de 30%.

$^1\text{H}$  RMN ( $\text{CDCl}_3$ ) :  $\delta$  (ppm) 1,42 (s, 36H,  $\text{C}(\text{CH}_3)_3$ ), 1,45 (m, 4H,  $\text{CH}_2$ ), 1,60 (m, 4H,  $\text{CH}_2$ ), , 3,04-3,33 (m, 12H,  $\text{CH}_2$ ), 3,91 (s, 2H,  $\text{NCH}_2\text{COO}$ ).

*b) Synthèse du 15-hydroxypentadécanoate de méthyle*

20 A 10 g de pentadécalactone (41,60 mmol) dans 41,60  $\text{cm}^3$  de méthanol, on ajoute 6,66  $\text{cm}^3$  de méthylate de sodium 2N (13,31 mmol) à 0°C.  
Après 9 heures, on ajoute 9,24  $\text{cm}^3$  d'acide acétique et on laisse agir pendant 15 minutes. Puis on évapore la solution sous vide à sec, on reprend ensuite par du dichlorométhane, et on effectue un lavage au bicarbonate de sodium. On sèche la  
25 phase organique obtenue par du sulfate de magnésium et on évapore le solvant à l'évaporateur rotatif. La purification se fait dans un mélange hexane/acétate d'éthyle 6:4. On obtient le 1-ol-pentadécanoate de méthyle avec un rendement de 80 %.

$^1\text{H}$  RMN ( $\text{CDCl}_3$ ) :  $\delta$  (ppm) 1,26 (m, 12H,  $(\text{CH}_2)_{10}$ ), 1,5-1,6 (m, 4H, H-2 et H-13), 2,30 (t, 2H,  $J = 7,60$  Hz, H-14), 3,64 (t, 1H,  $J = 5,84$  Hz, H-1), 3,67 (s, 3H, H-16).

*c) Synthèse du 2,3,4,6-tétra-O-acétyl- $\alpha$ -D-mannopyranoside de pentadécanoate de méthyle*

- 5 A  $0^\circ\text{C}$ , on ajoute  $5,26\text{ cm}^3$  de chlorure d'étain (44,94 mmol) à 8,72 g de mannose pentaacétylé (22,47 mmol) dans  $56\text{ cm}^3$  de dichlorométhane pendant 30 minutes. Puis on ajoute 7,34 g de 1-ol pentadécanoate de méthyle précédemment obtenu en a) (26,96 mmol). Après 2 heures, le mélange réactionnel est dilué par de l'éther éthylique et on verse dans une solution d'hydrogénophosphate de sodium ( $\text{NaHPO}_4$ ).
- 10 Les phases aqueuses sont extraites avec du diéther et les phases organiques sont successivement lavées par une solution de carbonate de sodium, de la saumure, puis séchées sur sulfate de magnésium. Le produit obtenu après évaporation sous vide à sec est purifié par chromatographie moyenne pression dans un mélange heptane/acétate d'éthyle 7:3. Le rendement est de 53 %.
- 15  $^1\text{H}$  RMN ( $\text{CDCl}_3$ ) :  $\delta$  (ppm) 1,26 (m, 20H,  $(\text{CH}_2)_{10}$ ), 1,59 (m, 4H,  $\text{OCH}_2\text{CH}_2$  et H-13), 2,01, 2,05, 2,12 et 2,17 (s, 3H,  $\text{OCOCH}_3$ ), 2,29 (t, 2H,  $J = 7,62$  Hz, H-14), 3,40 (m, 1H,  $J = 7,89$  Hz,  $\text{OCH}_a\text{CH}_2$ ), 3,66 (m, 1H,  $J = 7,89$  Hz,  $\text{OCH}_b\text{CH}_2$ ), 3,67 (s, 3H,  $\text{COOCH}_3$ ), 4,05 (ddd, 1H,  $J = 9,56$  Hz et  $5,57$  Hz, H-5), 4,1 (dd, 1H,  $J = 5,57$  Hz et  $12,32$  Hz, H-6a), 4,29 (dd, 1H,  $J = 5,57$  Hz et  $12,32$  Hz, H-6b), 4,8 (d, 1H,  $J = 1,85$
- 20 Hz, H-1), 5,23 (dd, 1H,  $J = 1,85$  Hz et  $3,23$  Hz, H-2), 5,27 (dd, 1H,  $J = 9,97$  Hz et  $9,56$  Hz, H-4), 5,35 (dd, 1H,  $J = 9,97$  Hz et  $3,23$  Hz, H-3).

*d) Synthèse de l' $\alpha$ -D-mannopyranoside de pentadécanoate de méthyle*

- On traite 3,63 g de produit obtenu à l'étape précédente (6,01 mmol) en solution dans  $12\text{ cm}^3$  de méthanol par  $3\text{ cm}^3$  de méthylate de sodium 2N (6,01 mmol). Lorsque la
- 25 réaction terminée, on neutralise cette dernière avec de l'Amberlite IR120 (1 équivalent poids/volume), on filtre et on évapore à sec sous vide.

$^1\text{H}$  RMN ( $\text{CDCl}_3$ ) :  $\delta$  (ppm) 1,28 (m, 20H,  $(\text{CH}_2)_{10}$ ), 1,59 (m, 4H,  $\text{OCH}_2\text{CH}_2$  et H-13), 2,34 (t, 2H,  $J = 7,62$  Hz, H-14), 3,41 (m, 1H,  $J = 6,71$  Hz,  $\text{OCH}_a\text{CH}_2$ ), 3,74 (m,

1H,  $J = 6,71$  Hz,  $\text{OCH}_b\text{CH}_2$ ), 3,67 (s, 3H,  $\text{CH}_3\text{OCO}$ ), 3,5-3,82 (m, 6H, H-2, H-3, H-4, H-5 et H-6), 4,75 (d, 1H,  $J = 1,82$  Hz, H-1).

*e) Synthèse du 2,3,4,6-tétra-O-benzyl- $\alpha$ -D-mannopyranoside de pentadécanoate de méthyle*

- 5 A 2 g (4,56 mmol) de produit obtenu à l'étape précédente d) en solution dans 20 cm<sup>3</sup> de diméthylformamide (DMF) anhydre, on ajoute successivement 4,54 g d'iodure de potassium (27,36 mmol), 1,09 g d'hydruure de sodium 60% (27,36 mmol) et 3,25 cm<sup>3</sup> de bromure de benzyle (27,36 mmol). Après 12 heures, on ajoute 18,24 cm<sup>3</sup> d'une solution saturée de chlorure d'ammonium, et on laisse agir pendant 10 minutes. Puis,
- 10 on dilue avec de l'eau et on extrait la phase organique par de l'acétate d'éthyle. Celle-ci est ensuite lavée avec de l'eau et de la saumure, et est finalement séchée par du sulfate de magnésium. On effectue par ailleurs un lavage supplémentaire par une solution saturée de thiosulfate de sodium afin d'éliminer les ions iodures. On évapore sous vide et on purifie l'huile résultante dans un mélange heptane/acétate d'éthyle
- 15 9:1. Le produit est obtenu avec un rendement de 60 %.

- <sup>1</sup>H RMN ( $\text{CDCl}_3$ ) : d (ppm) 1,28 (m, 20H,  $(\text{CH}_2)_{10}$ ), 1,49 (m, 2H,  $\text{OCH}_2\text{CH}_2$ ), 1,59 (m, 2H, H-13), 2,31 (t, 2H,  $J = 7,62$  Hz, H-14), 3,34 (m, 1H,  $J = 6,71$  Hz,  $\text{OCH}_a\text{CH}_2$ ), 3,63 (m, 1H,  $J = 6,71$  Hz,  $\text{OCH}_b\text{CH}_2$ ), 3,67 (s, 3H,  $\text{CH}_3\text{OCO}$ ), 3,75 (m, 1H,  $J = 8,97$  Hz et 6,21 Hz, H-5), 3,78 (s, 2H,  $\text{CH}_2\text{Phe}$ ), 3,90 (dd, 1H,  $J = 6,21$  Hz et  $J = 11,82$  Hz, H-6a), 3,97 (dd, 1H,  $J = 6,21$  Hz et  $J = 11,82$  Hz, H-6b), 4,07 (s, 2H,  $\text{CH}_2\text{Phe}$ ), 4,52 (dd,  $J = 2,91$  Hz et 7,83 Hz, H-3), 4,57 (s, 2H,  $\text{CH}_2\text{Phe}$ ), 4,63 (s, 2H,  $\text{CH}_2\text{Phe}$ ), 4,69 (dd, 1H,  $J = 2,52$  Hz et 2,91 Hz, H-2), 4,74 (1H,  $J = 2,52$  Hz, H-1), 4,85 (dd, 1H,  $J = 7,83$  Hz et 8,97 Hz, H-4), 7,35 (m, 20H, Phe).
- 20

- f) Synthèse du 2,3,4,6-tétra-O-benzyl- $\alpha$ -D-mannopyranoside de l'acide pentadécanoïque*
- 25

A 0,50 g (0,73 mmol) de produit obtenu à l'étape précédente e) en solution dans 7 cm<sup>3</sup> de méthanol, on ajoute 4,68 cm<sup>3</sup> d'une solution de soude 25 %. Le mélange réactionnel est chauffé à reflux pendant 30 minutes. Puis, on neutralise le mélange à



froid avec une solution d'acide chlorhydrique 5 %. On extrait la phase organique par de l'acétate d'éthyle, et on évapore à sec sous vide. La purification se fait dans un mélange heptane/acétate d'éthyle 4:6. Le produit est obtenu avec un rendement de 62 %.

- 5 **<sup>1</sup>H RMN (CDCl<sub>3</sub>)** : δ (ppm) 1,28 (m, 20H, (CH<sub>2</sub>)<sub>10</sub>), 1,49 (m, 2H, OCH<sub>2</sub>CH<sub>2</sub>), 1,59 (m, 2H, H-13), 2,34 (t, 2H, *J* = 7,62 Hz, H-14), 3,34 (m, 1H, *J* = 6,71 Hz, OCH<sub>a</sub>CH<sub>2</sub>), 3,63 (m, 1H, *J* = 6,71 Hz, OCH<sub>b</sub>CH<sub>2</sub>), 3,75 (m, 1H, *J* = 8,97 Hz et 6,21 Hz, H-5), 3,78 (s, 2H, CH<sub>2</sub>Phe), 3,90 (dd, 1H, *J* = 6,21 Hz et *J* = 11,82 Hz, H-6a), 3,97 (dd, 1H, *J* = 6,21 Hz et *J* = 11,82 Hz, H-6b), 4,07 (s, 2H, CH<sub>2</sub>Phe), 4,52 (dd, *J* = 2,91 Hz et 7,83 Hz, H-3), 4,57 (s, 2H, CH<sub>2</sub>Phe), 4,63 (s, 2H, CH<sub>2</sub>Phe), 4,69 (dd, 1H, *J* = 2,52 Hz et 2,91 Hz, H-2), 4,74 (1H, *J* = 2,52 Hz, H-1), 4,85 (dd, 1H, *J* = 7,83 Hz et 8,97 Hz, H-4), 7,35 (m, 20H, Phe).

*g) Synthèse du 2,3,4,6-tétra-O-benzyl-α-D-mannopyranoside de N-octadécyl-15-carbamoyl-pentadécanyle*

- 15 A 0,29 g (0,37 mmol) d'une solution de produit obtenu à l'étape précédente f), en solution dans 5 cm<sup>3</sup> de chloroforme, on ajoute successivement du 0,23 g de BOP (0,52 mmol), 0,21 cm<sup>3</sup> de diisopropyléthylamine (1,48 mmol) et 0,12 g d'octadécylamine (0,44 mmol). Lorsque la réaction est achevée, on dilue par du dichlorométhane et on effectue un lavage par de l'eau. Puis, on sèche par du sulfate de magnésium et on évapore à sec sous vide. Le produit obtenu est purifié par chromatographie moyenne pression dans un mélange heptane/acétate d'éthyle 6:4. Le produit est obtenu avec un rendement de 98 %.

- 25 **<sup>1</sup>H RMN (CDCl<sub>3</sub>)** : δ (ppm) 0,88 (t, 3H, *J* = 6,36 Hz, H-33), 1,27 (m, 50H, (CH<sub>2</sub>)<sub>25</sub>), 1,47 (m, 4H, OCH<sub>2</sub>CH<sub>2</sub> et H-17), 1,58 (m, 2H, H-13), 2,13 (t, 2H, *J* = 7,92 Hz, H-14), 3,23 (m, 2H, H-16), 3,34 (m, 1H, *J* = 6,71 Hz, OCH<sub>a</sub>CH<sub>2</sub>), 3,63 (m, 1H, *J* = 6,71 Hz, OCH<sub>b</sub>CH<sub>2</sub>), 3,75 (m, 1H, *J* = 8,97 Hz et 6,21 Hz, H-5), 3,78 (s, 2H, CH<sub>2</sub>Phe), 3,90 (dd, 1H, *J* = 6,21 Hz et *J* = 11,82 Hz, H-6a), 3,97 (dd, 1H, *J* = 6,21 Hz et *J* = 11,82 Hz, H-6b), 4,07 (s, 2H, CH<sub>2</sub>Phe), 4,52 (dd, *J* = 2,91 Hz et 7,83 Hz, H-3), 4,57 (s, 2H, CH<sub>2</sub>Phe), 4,63 (s, 2H, CH<sub>2</sub>Phe), 4,69 (dd, 1H, *J* = 2,52 Hz et 2,91 Hz, H-2), 4,74 (1H,

$J = 2,52$  Hz, H-1), 4,85 (dd, 1H,  $J = 7,83$  Hz et 8,97 Hz, H-4), 5,37 (bande, 1H, HNCO), 7,35 (m, 20H, Phe).

*h) Synthèse du 2,3,4,6-tétra-O-benzyl- $\alpha$ -D-mannopyranoside de 15-octadécylamino-pentadécanyle*

5 A 0,77 g (0,75 mmol) de produit obtenu à l'étape précédente g), dans 15 cm<sup>3</sup> de tétrahydrofurane (THF) anhydre, on ajoute 0,056 g d'hydruure de lithium aluminium  $AlLiH_4$  (1,50 mmol). On chauffe à reflux pendant 10 heures. Puis, le mélange réactionnel est refroidi dans un bain de glace et on ajoute 56  $\mu$ l d'eau, puis 112  $\mu$ l de soude 2N après 10 minutes, et enfin encore 56  $\mu$ l d'eau 10 minutes plus tard. On filtre  
10 et on évapore à sec sous vide. Le produit obtenu est purifié dans un mélange de dichlorométhane/méthanol/ammoniac 28 % 9:2:0,5. Le produit est obtenu avec un rendement de 86 %.

<sup>1</sup>H RMN ( $CDCl_3$ ) :  $\delta$  (ppm) 0,88 (t, 3H,  $J = 6,36$  Hz, H-33), 1,27 (m, 50H,  $(CH_2)_{25}$ ), 1,4-1,6 (m, 9H,  $OCH_2CH_2$ , H-17, H-14, H-17et NH), 2,57 (t, 4H,  $J = 7,92$  Hz, H-15  
15 et H-16), 3,34 (m, 1H,  $J = 6,71$  Hz,  $OCH_2CH_2$ ), 3,63 (m, 1H,  $J = 6,71$  Hz,  $OCH_2CH_2$ ), 3,75 (m, 1H,  $J = 8,97$  Hz et 6,21 Hz, H-5), 3,78 (s, 2H,  $CH_2Phe$ ), 3,90 (dd, 1H,  $J = 6,21$  Hz et  $J = 11,82$  Hz, H-6a), 3,97 (dd, 1H,  $J = 6,21$  Hz et  $J = 11,82$  Hz, H-6b), 4,07 (s, 2H,  $CH_2Phe$ ), 4,52 (dd,  $J = 2,91$  Hz et 7,83 Hz, H-3), 4,57 (s, 2H,  $CH_2Phe$ ), 4,63 (s, 2H,  $CH_2Phe$ ), 4,69 (dd, 1H,  $J = 2,52$  Hz et 2,91 Hz, H-2), 4,74 (1H,  
20  $J = 2,52$  Hz, H-1), 4,85 (dd, 1H,  $J = 7,83$  Hz et 8,97 Hz, H-4), 7,35 (m, 20H, Phe).

*i) Synthèse du 2,3,4,6-tétra-O-benzyl- $\alpha$ -D-mannopyranoside de (3-[4-(3-tert-butoxycarbonyl-amino-propyl-tert-butoxycarbonyl-amino)-butyl-tert-butoxycarbonyl-amino]-méthylène-carbamoyl)-15-pentadécanyl-16-octadécyle*

A 0,63 g (0,61 mmol) d'une solution de produit obtenu précédemment à l'étape h),  
25 dans 10 cm<sup>3</sup> de chloroforme, on ajoute successivement 0,38 g de BOP (0,85 mmol), 0,425 cm<sup>3</sup> de diisopropyléthylamine (2,44 mmol) et 0,48 g d'acide 3-[4-(3-tert-butoxycarbonyl-amino-propyl-tert-butoxycarbonyl-amino)-butyl-tert-butoxycarbonyl-amino]-acétique (FRM 375) (0,73 mmol) obtenu à l'étape a). Au bout de 4 heures, on

dilue par du dichlorométhane et on effectue un lavage à l'eau. On sèche par du sulfate de magnésium et on évapore à sec sous vide. Le produit obtenu est purifié par chromatographie moyenne pression dans un mélange heptane/acétate d'éthyle 6:4. Le produit est obtenu avec un rendement de 80 %.

- 5 **<sup>1</sup>H RMN (CDCl<sub>3</sub>)** : δ (ppm) 0,88 (t, 3H, *J*= 6,36 Hz, H-33), 1,27 (m, 50H, (CH<sub>2</sub>)<sub>25</sub>), 1,4-1,6 (m, 17H, OCH<sub>2</sub>CH<sub>2</sub>, H-17, H-14, H-17, H-37, H-40, H-41 et H-44), 1,46 (m, 36H, Boc), 2,8-2,9 (m, 6H, H-15, H-16 et H-35), 3,09-3,33 (m, 12H, H-36, H-38, H-39, H-42, H-43 et H-45), 3,34 (m, 1H, *J*= 6,71 Hz, OCH<sub>a</sub>CH<sub>2</sub>), 3,63 (m, 1H, *J*= 6,71 Hz, OCH<sub>b</sub>CH<sub>2</sub>), 3,75 (m, 1H, *J*= 8,97 Hz et 6,21 Hz, H-5), 3,78 (s, 2H, CH<sub>2</sub>Phe), 10 3,90 (dd, 1H, *J*= 6,21 Hz et *J*= 11,82 Hz, H-6a), 3,97 (dd, 1H, *J*= 6,21 Hz et *J*= 11,82 Hz, H-6b), 4,07 (s, 2H, CH<sub>2</sub>Phe), 4,52 (dd, *J*= 2,91 Hz et 7,83 Hz, H-3), 4,57 (s, 2H, CH<sub>2</sub>Phe), 4,63 (s, 2H, CH<sub>2</sub>Phe), 4,69 (dd, 1H, *J*= 2,52 Hz et 2,91 Hz, H-2), 4,74 (1H, *J*= 2,52 Hz, H-1), 4,85 (dd, 1H, *J*= 7,83 Hz et 8,97 Hz, H-4), 7,35 (m, 18H, Phe).

- 15 *j) Synthèse de l'α-D-mannopyranoside de (3-[4-(3-tert-butoxycarbonyl-amino-propyl-tert-butoxycarbonyl-amino)-butyl-tert-butoxycarbonyl-amino]-méthylène-carbamoyl)-15-pentadécanyl-16-octadécyle*

- A 0,63 g (0,38 mmol) de produit obtenu à l'étape précédente i), dans 5 cm<sup>3</sup> de méthanol, on ajoute 10 % de palladium sur charbon (0,027 g). La solution est agitée sous pression d'hydrogène à température ambiante. Au bout de 6 heures, on filtre puis 20 on évapore à sec sous vide. La réaction est quantitative.

- <sup>1</sup>H RMN (CD<sub>3</sub>OD)** : δ (ppm) 0,88 (t, 3H, *J*= 6,36 Hz, H-33), 1,27 (m, 50H, (CH<sub>2</sub>)<sub>25</sub>), 1,4-1,6 (m, 17H, OCH<sub>2</sub>CH<sub>2</sub>, H-17, H-14, H-17, H-37, H-40, H-41 et H-44), 1,46 (m, 36H, Boc), 2,8-2,9 (m, 6H, H-15, H-16 et H-35), 3,09-3,33 (m, 12H, H-36, H-38, H-39, H-42, H-43 et H-45), 3,34 (m, 1H, *J*= 6,71 Hz, OCH<sub>a</sub>CH<sub>2</sub>), 3,5-3,82 25 (m, 6H, H-2, H-3, H-4, H-5 et H-6), 3,63 (m, 1H, *J*= 6,71 Hz, OCH<sub>b</sub>CH<sub>2</sub>), 4,72 (1H, *J*= 2,52 Hz, H-1).

- k) Synthèse de l'α-D-mannopyranoside de (3-[4-(3-amino-propyl-amino)-butyl-amino]-méthylène-carbamoyl)-15-pentadécanyl-16-octadécyle (composé 1)*

A 0,37 g (0,28 mmol) de produit obtenu à l'étape précédente j), on ajoute 21,50 cm<sup>3</sup> de tétrahydrofurane (THF) distillé. Après 1 heure, le mélange réactionnel est concentré à froid et lyophilisé. On vérifie le degré de pureté du produit en solution dans du méthanol par CLHP comme décrit dans la partie "Matériel et Méthodes".

- 5 <sup>1</sup>H RMN (CD<sub>3</sub>OD) : 0,88 (t, 3H,  $J = 6,36$  Hz, H-33), 1,27 (m, 14H, (CH<sub>2</sub>)<sub>25</sub>), 1,4-1,6 (m, 17H, OCH<sub>2</sub>CH<sub>2</sub>, H-17, H-14, H-17, H-37, H-40, H-41 et H-44), 2,8-2,9 (m, 6H, H-15, H-16 et H-35), 2,92 (m, 2H, H-45), 2,92-3,17 (m, 12H, H-36, H-38, H-39, H-42, H-43), 3,34 (m, 1H,  $J = 6,71$  Hz, OCH<sub>a</sub>CH<sub>2</sub>), 3,5-3,82 (m, 6H, H-2, H-3, H-4, H-5 et H-6), 3,63 (m, 1H,  $J = 6,71$  Hz, OCH<sub>b</sub>CH<sub>2</sub>), 4,72 (1H,  $J = 2,02$  Hz, H-1).

10 **Exemple 2 : synthèse du 6-désoxy- $\alpha$ -L-mannopyranoside de (3-[4-(3-amino-propyl-amino)-butyl-amino]-méthylène-carbamoyl)-15-pentadécanyl-16-octadécyle (composé 2)**

*a) Synthèse de l'acide 3-[4-(3-tert-butoxycarbonyl-amino-propyl-tert-butoxycarbonyl-amino)-butyl-tert-butoxycarbonyl-amino] acétique (FRM 375)*

- 15 A une solution de spermine (5 g ; 24,96 mmoles) dans du méthanol (125 ml), on ajoute du cyanoborohydrure de sodium NaBH<sub>3</sub>CN (0,548 g ; 8,74 mmoles). La solution est ensuite soumise à une vive agitation. Par l'intermédiaire d'une ampoule isobare, on ajoute en 100 minutes une solution d'acide glyoxylique (2,34 g ; 25,46 mmoles) dans du méthanol (80 ml). Après une nuit, on ajoute au mélange de la
- 20 triéthylamine (3,86 ml ; 27,71 mmoles) et du di-tert-butyl dicarbonate (27,67 g ; 129,79 mmoles) solubilisé dans du tétrahydrofurane (55 ml). Après une nuit, on concentre à l'évaporateur rotatif puis on reprend par de l'acétate d'éthyle (63 ml) et on effectue un lavage à l'hydrogénosulfate de potassium et à la saumure. Puis on sèche sur sulfate de magnésium et on concentre. Le produit obtenu est purifiée par
- 25 chromatographie (CH<sub>2</sub>Cl<sub>2</sub>/MeOH 9 : 1). Le rendement est de 30%.

<sup>1</sup>H RMN (CDCl<sub>3</sub>) :  $\delta$  (ppm) 1,42 (s, 36H, C(CH<sub>3</sub>)<sub>3</sub>), 1,45 (m, 4H, CH<sub>2</sub>), 1,60 (m, 4H, CH<sub>2</sub>), , 3,04-3,33 (m, 12H, CH<sub>2</sub>), 3,91 (s, 2H, NCH<sub>2</sub>COO).

*b) Synthèse du 15-hydroxy-pentadécanoate de méthyle*

A 10 g de pentadécalactone (41,60 mmol) dans 41,60 cm<sup>3</sup> de méthanol, on ajoute 6,66 cm<sup>3</sup> de méthylate de sodium 2N (13,31 mmol) à 0°C. Après 9 heures, on ajoute 9,24 cm<sup>3</sup> d'acide acétique et on laisse agir pendant 15 minutes. Puis on évapore à sec sous vide la solution qui est ensuite reprise par du dichlorométhane, et on effectue un lavage au bicarbonate de sodium. On sèche la phase organique obtenue par du sulfate de magnésium et on évapore le solvant à l'évaporateur rotatif. La purification se fait dans un mélange hexane/acétate d'éthyle 6:4. On obtient le 1-ol-pentadécanoate de méthyle avec un rendement de 80 %.

10 <sup>1</sup>H RMN (CDCl<sub>3</sub>) : δ (ppm) 1,26 (m, 12H, (CH<sub>2</sub>)<sub>10</sub>), 1,5-1,6 (m, 4H, H-2 et H-13), 2,30 (t, 2H, J = 7.60 Hz, H-14), 3,64 (t, 1H, J = 5.84 Hz, H-1), 3,67 (s, 3H, H-16).

*c) Synthèse du 2,3,4-tri-O-acétyl-6-désoxy-α-L-mannopyranoside de pentadécanoate de méthyle*

A 0°C, on ajoute 2,49 cm<sup>3</sup> de chlorure d'étain (21,30 mmol) à 3,55 g de rhamnose tétraacétylé (10,65 mmol) dans 27 cm<sup>3</sup> de dichlorométhane pendant 30 minutes. Puis on additionne 3,48 g de 1-ol pentadécanoate de méthyle précédemment obtenu (12,78 mmol). Après 2 heures, le mélange réactionnel est dilué par de l'éther éthylique et on verse dans une solution de phosphate de sodium (Na<sub>2</sub>PO<sub>4</sub>). Les phases aqueuses sont extraites avec du diéthyléther et les phases organiques sont successivement lavées par une solution de carbonate de sodium, de la saumure, puis séchées par du sulfate de magnésium. Après évaporation à sec sous vide, on purifie par chromatographie moyenne pression dans un mélange heptane/acétate d'éthyle 7:3. Le produit est obtenu avec un rendement de 60 %.

25 <sup>1</sup>H RMN (CDCl<sub>3</sub>) : δ (ppm) 1,20 (d, 3H, J = 6,45 Hz, H-6), 1,26 (m, 20H, (CH<sub>2</sub>)<sub>10</sub>), 1,59 (m, 4H, OCH<sub>2</sub>CH<sub>2</sub> et H-13), 1,98, 2,04 et 2,16 (s, 3H, OCOCH<sub>3</sub>), 2,29 (t, 2H, J = 7,62 Hz, H-14), 3,40 (m, 1H, J = 6,71 Hz, OCH<sub>a</sub>CH<sub>2</sub>), 3,66 (m, 1H, J = 6,71 Hz, OCH<sub>b</sub>CH<sub>2</sub>), 3,67 (s, 3H, COOCH<sub>3</sub>), 3,88 (m, 1H, J = 6,45 Hz et 9,97 Hz, H-5), 4,70 (d, 1H, J = 1,72 Hz, H-1), 5,06 (dd, 1H, J = 9,97 Hz et 9,97 Hz, H-4), 5,22 (dd, 1H, J = 1,72 Hz et 3,52 Hz, H-2), 5,30 (dd, 1H, J = 3,52 Hz et 9,97 Hz, H-3).

*d) Synthèse de l' $\alpha$ -désoxy-L-6-mannopyranoside de pentadécanoate de méthyle*

On traite 5,08 g de produit obtenu à l'étape c) (9,34 mmol) en solution dans 20 cm<sup>3</sup> de méthanol par 9,34 ml de méthylate de sodium 2N (18,68 mmol). Lorsque la réaction est achevée, on neutralise le mélange réactionnel avec de l'Amberlite IR120, on filtre et on évapore à sec sous vide.

<sup>1</sup>H RMN (CDCl<sub>3</sub>) :  $\delta$  (ppm) 1,20 (d, 3H,  $J = 6,45$  Hz, H-6), 1,26 (m, 20H, (CH<sub>2</sub>)<sub>10</sub>), 1,59 (m, 4H, OCH<sub>2</sub>CH<sub>2</sub> et H-13), 2,29 (t, 2H,  $J = 7,62$  Hz, H-14), 3,40 (m, 1H,  $J = 6,71$  Hz, OCH<sub>b</sub>CH<sub>2</sub>), 3,66 (m, 1H,  $J = 6,71$  Hz, OCH<sub>b</sub>CH<sub>2</sub>), 3,67 (s, 3H, CH<sub>3</sub>OCO), 3,6-3,9 (m, 4H, H-2, H-3, H-4 et H-5), 4,70 (d, 1H,  $J = 1,72$  Hz, H-1).

*e) Synthèse du 2,3,4-tri-O-benzyl-6-désoxy- $\alpha$ -L-mannopyranoside de pentadécanoate de méthyle*

A 2,09 g (5,00 mmol) de produit obtenu à l'étape précédente d), dans 30 cm<sup>3</sup> de diméthylformamide (DMF) anhydre, on ajoute successivement 3,32 g d'iodure de potassium (20,00 mmol), 0,80 g d'hydruure de sodium 60 % (20,00 mmol) et 2,38 cm<sup>3</sup> du bromure de benzyle (20,00 mmol). Après 12 heures, on ajoute 20 cm<sup>3</sup> d'une solution saturée de chlorure d'ammonium et on laisse agir pendant 10 minutes. Puis, on dilue avec de l'eau et on extrait la phase organique par de l'acétate d'éthyle. Celle-ci est ensuite lavée avec de l'eau et de la saumure, avant d'être finalement séchée par du sulfate de magnésium.

Par ailleurs, un lavage supplémentaire par une solution saturée de thiosulfate de sodium est effectué afin d'éliminer les ions iodures. On évapore à sec sous vide et on purifie l'huile résultante dans un mélange heptane/acétate d'éthyle 9:1. Le produit est obtenu avec un rendement de 60 %.

<sup>1</sup>H RMN (CDCl<sub>3</sub>) :  $\delta$  (ppm) 1,28 (m, 20H, (CH<sub>2</sub>)<sub>10</sub>), 1,33 (d, 3H,  $J = 6,21$  Hz, H-6), 1,59 (m, 4H, OCH<sub>2</sub>CH<sub>2</sub> et H-13), 2,31 (t, 2H,  $J = 7,62$  Hz, H-14), 3,40 (m, 1H,  $J = 6,71$  Hz, OCH<sub>a</sub>CH<sub>2</sub>), 3,61 (dd, 1H,  $J = 8,96$  Hz et 9,5 Hz, H-4), 3,66 (m, 1H,  $J = 6,71$  Hz, OCH<sub>b</sub>CH<sub>2</sub>), 3,67 (s, 3H, CH<sub>3</sub>OCO), 3,68 (m, 1H,  $J = 9,5$  Hz et 6,21 Hz, H-5), 3,75 (dd, 1H,  $J = 2,01$  Hz et 3,02 Hz, H-2), 3,88 (dd,  $J = 3,02$  Hz et 8,96 Hz, H-3), 4,64 (s, 6H, CH<sub>2</sub>Phe), 4,73 (1H,  $J = 2,01$  Hz, H-1), 7,35 (m, 15H, Phe).

*f) Synthèse du 2,3,4-tri-O-benzyl-6-désoxy- $\alpha$ -L-mannopyranoside de l'acide pentadécanoïque*

A 0,50 g (0,73 mmol) d'une solution de produit obtenu à l'étape précédente e), dans 7 cm<sup>3</sup> de méthanol, on ajoute 4,68 ml de soude 25 %. Le mélange réactionnel est  
5 chauffé à reflux pendant 30 minutes. Puis, on neutralise le mélange à froid avec une solution d'acide chlorhydrique 5 %. On extrait la phase organique par de l'acétate d'éthyle et on évapore à sec sous vide. La purification se fait dans un mélange heptane/acétate d'éthyle 4:6. Le produit est obtenu avec un rendement de 72 %.

<sup>1</sup>H RMN (CDCl<sub>3</sub>) :  $\delta$  (ppm) 1,28 (m, 20H, (CH<sub>2</sub>)<sub>10</sub>), 1,33 (d, 3H,  $J$ = 6,21 Hz, H-6),  
10 1,59 (m, 4H, OCH<sub>2</sub>CH<sub>2</sub> et H-13), 2,34 (t, 2H,  $J$ = 7,62 Hz, H-14), 3,40 (m, 1H,  $J$ = 6,71 Hz, OCH<sub>a</sub>CH<sub>2</sub>), 3,61 (dd, 1H,  $J$ = 8,96 Hz et 9,5 Hz, H-4), 3,66 (m, 1H,  $J$ = 6,71 Hz, OCH<sub>b</sub>CH<sub>2</sub>), 3,68 (m, 1H,  $J$ = 9,5 Hz et 6,21 Hz, H-5), 3,75 (dd, 1H,  $J$ = 2,01 Hz et 3,02 Hz, H-2), 3,88 (dd,  $J$ = 3,02 Hz et 8,96 Hz, H-3), 4,64 (s, 6H, CH<sub>2</sub>Phe), 4,73 (1H,  $J$ = 2,01 Hz, H-1), 7,35 (m, 20H, Phe).

15 *g) Synthèse du 2,3,4-tri-O-benzyl-6-désoxy- $\alpha$ -L-mannopyranoside de N-octadécyl-15-carbamoyl-pentadécanyle*

A 0,70 g (1,04 mmol) d'une solution de produit précédemment obtenu à l'étape f), dans 13 cm<sup>3</sup> de chloroforme, on ajoute successivement 0,69 g de BOP (1,56 mmol), 0,72 cm<sup>3</sup> de diisopropyléthylamine (4,16 mmol) et 0,34 g d'octadécylamine  
20 (1,25 mmol). Lorsque la réaction est terminée, on dilue par du dichlorométhane, on effectue un lavage à l'eau, on sèche sur sulfate de magnésium, et on évapore à sec sous vide. Le produit obtenu est purifié par chromatographie moyenne pression dans un mélange heptane/acétate d'éthyle 6:4. Le produit est obtenu avec un rendement de 84 %.

25 <sup>1</sup>H RMN (CDCl<sub>3</sub>) :  $\delta$  (ppm) 0,88 (t, 3H,  $J$ = 6,36 Hz, H-33), 1,27 (m, 50H, (CH<sub>2</sub>)<sub>25</sub>), 1,33 (d, 3H,  $J$ = 6,21 Hz, H-6), 1,47 (m, 4H, OCH<sub>2</sub>CH<sub>2</sub> et H-17), 1,58 (m, 2H, H-13), 2,13 (t, 2H,  $J$ = 7,92 Hz, H-14), 3,23 (m, 2H, H-16), 3,40 (m, 1H,  $J$ = 6,71 Hz, OCH<sub>a</sub>CH<sub>2</sub>), 3,61 (dd, 1H,  $J$ = 8,96 Hz et 9,5 Hz, H-4), 3,66 (m, 1H,  $J$ = 6,71 Hz, OCH<sub>b</sub>CH<sub>2</sub>), 3,68 (m, 1H,  $J$ = 9,5 Hz et 6,21 Hz, H-5), 3,75 (dd, 1H,  $J$ = 2,01 Hz et

3,02 Hz, H-2), 3,88 (dd,  $J = 3,02$  Hz et 8,96 Hz, H-3), 4,64 (s, 6H,  $\text{CH}_2\text{Phe}$ ), 4,73 (1H,  $J = 2,01$  Hz, H-1), 5,37 (bande, 1H,  $\text{HNCO}$ ), 7,35 (m, 15H, Phe).

*h) Synthèse du 2,3,4-tri-O-benzyl-6-désoxy- $\alpha$ -L-mannopyranoside de 15-octadécyl-amino-pentadécanyle*

- 5 A 0,81 g (0,86 mmol) de produit obtenu à l'étape précédente g), dans 15 cm<sup>3</sup> de tétrahydrofurane (THF) anhydre, on ajoute 0,065 g d'hydruure de lithium; aluminium  $\text{AlLiH}_4$  (1,72 mmol), et on chauffe à reflux pendant 10 heures. Puis, le mélange réactionnel est refroidi dans de un bain de glace et on ajoute 65  $\mu\text{l}$  d'eau, puis 130  $\mu\text{l}$  de soude 2N au bout de 10 minutes, et enfin à nouveau 65  $\mu\text{l}$  d'eau après 10 minutes.
- 10 On filtre et on évapore à sec sous vide. La purification se fait dans un mélange dichlorométhane/méthanol/ammoniac 28% 9:2:0,5. Le produit est obtenu avec un rendement de 93 %.

<sup>1</sup>H RMN ( $\text{CDCl}_3$ ) :  $\delta$  (ppm) 0,88 (t, 3H,  $J = 6,36$  Hz, H-33), 1,27 (m, 50H,  $(\text{CH}_2)_{25}$ ), 1,33 (d, 3H,  $J = 6,21$  Hz, H-6), 1,4-1,6 (m, 9H,  $\text{OCH}_2\text{CH}_2$ , H-17, H-14, H-17et NH), 2,57 (t, 4H,  $J = 7,92$  Hz, H-15 et H-16), 3,40 (m, 1H,  $J = 6,71$  Hz,  $\text{OCH}_a\text{CH}_2$ ), 3,61 (dd, 1H,  $J = 8,96$  Hz et 9,5 Hz, H-4), 3,66 (m, 1H,  $J = 6,71$  Hz,  $\text{OCH}_b\text{CH}_2$ ), 3,68 (m, 1H,  $J = 9,5$  Hz et 6,21 Hz, H-5), 3,75 (dd, 1H,  $J = 2,01$  Hz et 3,02 Hz, H-2), 3,88 (dd,  $J = 3,02$  Hz et 8,96 Hz, H-3), 4,64 (s, 6H,  $\text{CH}_2\text{Phe}$ ), 4,73 (1H,  $J = 2,01$  Hz, H-1), 7,35 (m, 15H, Phe).

20 *i) Synthèse du 2,3,4-tri-O-benzyl-6-désoxy- $\alpha$ -L-mannopyranoside de (3-[4-(3-tert-butoxycarbonyl-amino-propyl-tert-butoxycarbonyl-amino)-butyl-tert-*

*butoxycarbonyl-amino]-méthylène-carbamoyl)-15-pentadécanyl-16-octadécyle*

- A 0,78 g (0,86 mmol) d'une solution de produit obtenu à l'étape précédente h), en solution dans 7 cm<sup>3</sup> de chloroforme, on ajoute successivement 0,53 g de BOP (1,20 mmol), 0,30 cm<sup>3</sup> de diisopropyléthylamine (1,72 mmol) et 0,62 g de FRM 375 obtenu à l'étape a) (0,95 mmol). Après 4 heures, on dilue avec du dichlorométhane, on effectue un lavage à l'eau, on sèche sur sulfate de magnésium, et on évapore à sec
- 25



sous vide. Le produit obtenu est purifié par chromatographie "flash" dans un mélange heptane/acétate d'éthyle 6:4. Le produit est obtenu avec un rendement de 72 %.

<sup>1</sup>H RMN (CDCl<sub>3</sub>) : δ (ppm) 0,88 (t, 3H, *J* = 6,36 Hz, H-33), 1,27 (m, 50H, (CH<sub>2</sub>)<sub>25</sub>), 1,33 (d, 3H, *J* = 6,21 Hz, H-6), 1,4-1,6 (m, 17H, OCH<sub>2</sub>CH<sub>2</sub>, H-17, H-14, H-17, H-37, H-40, H-41 et H-44), 1,46 (m, 36H, Boc), 2,8-2,9 (m, 6H, H-15, H-16 et H-35), 3,09-3,33 (m, 12H, H-36, H-38, H-39, H-42, H-43 et H-45), 3,40 (m, 1H, *J* = 6,71 Hz, OCH<sub>a</sub>CH<sub>2</sub>), 3,65 (s, 2H, CH<sub>2</sub>Phe), 3,66 (m, 1H, *J* = 6,71 Hz, OCH<sub>b</sub>CH<sub>2</sub>), 3,68 (m, 1H, *J* = 9,5 Hz et 6,21 Hz, H-5), 3,99 (s, 2H, CH<sub>2</sub>Phe), 4,02 (dd, 1H, *J* = 8,96 Hz et 9,5 Hz, H-4), 4,32 (s, 2H, CH<sub>2</sub>Phe), 4,57 (dd, 1H, *J* = 2,01 Hz et 3,02 Hz, H-2), 4,73 (1H, *J* = 2,01 Hz, H-1), 4,82 (dd, *J* = 3,02 Hz et 8,96 Hz, H-3), 7,35 (m, 18H, Phe).

*j) Synthèse du 6-désoxy-α-L-mannopyranoside de (3-[4-(3-tert-butoxycarbonyl-amino-propyl-tert-butoxycarbonyl-amino)-butyl-tert-butoxycarbonyl-amino]-méthylène-carbamoyl)-15-pentadécanyl-16-octadécyle*

A 0,74 g (0,48 mmol) de produit obtenu à l'étape précédente i), en solution dans 10 cm<sup>3</sup> de méthanol, on ajoute 10 % (0,034 g) de palladium sur charbon. La solution est agitée sous pression d'hydrogène à température ambiante. Après 4 heures, on filtre puis on évapore à sec sous vide. La réaction est quantitative.

<sup>1</sup>H RMN (CD<sub>3</sub>OD) : δ (ppm) 0,88 (t, 3H, *J* = 6,36 Hz, H-33), 1,20 (d, 3H, *J* = 6,45 Hz, H-6), 1,27 (m, 14H, (CH<sub>2</sub>)<sub>25</sub>), 1,4-1,6 (m, 17H, OCH<sub>2</sub>CH<sub>2</sub>, H-17, H-14, H-17, H-37, H-40, H-41 et H-44), 1,46 (m, 36H, Boc), 2,8-2,9 (m, 6H, H-15, H-16 et H-35), 3,09-3,33 (m, 12H, H-36, H-38, H-39, H-42, H-43 et H-45), 3,40 (m, 1H, *J* = 6,71 Hz, OCH<sub>a</sub>CH<sub>2</sub>), 3,66 (m, 1H, *J* = 6,71 Hz, OCH<sub>b</sub>CH<sub>2</sub>), 3,6-3,9 (m, 4H, H-2, H-3, H-4 et H-5), 4,73 (1H, *J* = 2,01 Hz, H-1).

*k) Synthèse du 6-désoxy-α-L-mannopyranoside de (3-[4-(3-amino-propyl-amino)-butyl-amino]-méthylène-carbamoyl)-15-pentadécanyl-16-octadécyle (composé 2)*

A 0,40 g (0,31 mmol) de produit obtenu à l'étape précédente j), on ajoute 24 cm<sup>3</sup> de tétrahydrofurane (THF) distillé. Après 1 heure, le mélange réactionnel est évaporé sous vide à froid et à sec, puis est lyophilisé. On vérifie le degré de pureté du produit en solution dans du méthanol par CLHP.

<sup>1</sup>H RMN (CD<sub>3</sub>OD) : δ (ppm) 0,88 (t, 3H, J= 6,36 Hz, H-33), 1,20 (d, 3H, J= 6,45 Hz, H-6'), 1,27 (m, 14H, (CH<sub>2</sub>)<sub>25</sub>), 1,4-1,6 (m, 17H, OCH<sub>2</sub>CH<sub>2</sub>, H-17, H-14, H-17, H-37, H-40, H-41 et H-44), 2,8-2,9 (m, 6H, H-15, H-16 et H-35), 2,92 (m, 2H, H-45), 2,92-3,17 (m, 12H, H-36, H-38, H-39, H-42, H-43), 3,40 (m, 1H, J= 6,71 Hz, OCH<sub>a</sub>CH<sub>2</sub>), 3,66 (m, 1H, J= 6,71 Hz, OCH<sub>b</sub>CH<sub>2</sub>), 3,6-3,9 (m, 4H, H-2, H-3, H-4 et H-5), 4,73 (1H, J= 2,01 Hz, H-1).

**Exemple 3 : Synthèse du 6-désoxy-β-L-galactopyrannoside du 1-[-(3-[4-(3-amino-propyl-amino)-butyl-amino-propyl-amino]-méthylène-carbamoyl)-15-pentadécanyl-16-octadécanyle (composé 3)]**

- 10 a) *Synthèse de l'acide {3-[4-(3-benzyloxycarbonyl-amino-propyl-benzyloxycarbonyl-amino)-butyl-benzyloxycarbonyl-amino]-propylamino} acétique*
- A une solution de spermine (10 g ; 49,91 mmoles) dans du méthanol (200 ml), on ajoute du cyanoborohydrure de sodium NaBH<sub>3</sub>CN (1,10 g ; 17,47 mmoles). La solution est ensuite soumise à une vive agitation. Par l'intermédiaire d'une ampoule isobare, on ajoute en 100 minutes une solution d'acide glyoxylique (4,59 g ; 49,91 mmoles) dans du méthanol (120 ml). Après une nuit, on place le mélange réactionnel dans un bain de glace et on ajoute successivement de la soude 2N (34 ml) et du chloroformate de benzyle (14,25 ml ; 99,82 mmoles) en 10 portions. On mélange vigoureusement en maintenant le bain entre 5°C et 10°C. Au bout de 2 heures à
- 15 température ambiante, le mélange est extrait avec de l'éther et neutralisé par une solution d'acide chlorhydrique 5N. La phase organique est ensuite séchée sur sulfate de magnésium, et on concentre à l'évaporateur rotatif. Le produit obtenu est purifié par chromatographie (100 % CH<sub>2</sub>Cl<sub>2</sub> puis CH<sub>2</sub>Cl<sub>2</sub>/MeOH 9 : 1). Le rendement est de 52%.
- 20 <sup>1</sup>H RMN (CDCl<sub>3</sub>) : δ (ppm) 1,28 (t, 4H, CH<sub>2</sub>), 1,60 (m, 4H, CH<sub>2</sub>), , 3,04-3,33 (m, 12H, CH<sub>2</sub>), 3,49 (s, 2H, NCH<sub>2</sub>COO), 5,07 (s, 8H, CH<sub>2</sub>), 7,27 (m, 20H, Phe).

***b) Synthèse du 15-hydroxypentadécanoate de méthyle***

La pentadécalactone (10 g ; 41,6 mmol) en solution dans du méthanol (41,6 ml) est traitée par du méthylate de sodium 2N (6,656 ml ; 13,31 mmol) à 0°C. Après 9 heures, on ajoute 9,24 ml d'acide acétique et on laisse agir pendant 15 minutes. Puis on concentre la solution et l'huile résultante est dissoute dans du dichlorométhane et lavée par du bicarbonate de sodium. Après décantation, la phase organique est séchée sur sulfate de magnésium et on évapore. La purification se fait dans un mélange 6 : 4 hexane/acétate d'éthyle (AcOEt) pour donner le 15-hydroxypentadécanoate de méthyle avec un rendement de 80%.

**<sup>1</sup>H RMN (CDCl<sub>3</sub>) :** δ (ppm) 1,29 (m, 20H, (CH<sub>2</sub>)<sub>10</sub>), 1,5-1,6 (m, 4H, H-2 et H-13), 2,30 (t, 2H, *J*= 7.60 Hz, H-14), 3,64 (t, 1H, *J*= 5,84 Hz, H-1), 3,67 (s, 3H, H-16).

***c) Synthèse de la N-octadécyl-15-hydroxypentadécanamide***

On fond 10g de 15-hydroxypentadécanoate de méthyle obtenu à l'étape précédente b) (36,85 mmol) et 19,86 g d'octadécylamine (73,70 mmol) à 150°C sous vide. Au bout de 24 heures, le mélange est refroidi et dilué par du dichlorométhane. On obtient un précipité qui est filtré sur Büchner. Le solide obtenu est ensuite recristallisé dans le méthanol pour donner la N-octadécyl-15-hydroxypentadécanamide avec un rendement de 100 %.

**<sup>1</sup>H RMN (CDCl<sub>3</sub>) :** δ (ppm) 0,88 (t, 3H, *J*= 6,96 Hz, H-33), 1,26 (m, 54H, (CH<sub>2</sub>)<sub>27</sub>), 1,4-1,6 (m, 6H, H-2, H-13 et H-17), 2,30 (t, 2H, *J*= 7.60 Hz, H-14), 3,25 (m, 2H, H-16), 3,64 (t, 2H, *J*= 5,84 Hz, H-1), 5,39 (bande NHCO).

**<sup>13</sup>C RMN (CDCl<sub>3</sub>) :** δ (ppm) 14,48 (C-33), 25,3 et 26,3 (C-2 et C-13), 29,72 ((CH<sub>2</sub>)<sub>27</sub>), 36,7 et 34,8 (C-14 et C-16), 63,6 (C-1), 174,31 (CO).

***d) Synthèse du 15-octadécylamino-pentadécanol***

A une solution de 20 g de N-octadécyl-15-hydroxypentadécanamide obtenu à l'étape précédente c) (39,22 mmol) dans du tétrahydrofurane anhydre (250 ml), on ajoute 2,98 g d'hydruure de lithium aluminium LiAlH<sub>4</sub> (78,44 mmol). La réaction se fait à reflux pendant 10 heures. Après avoir refroidi le mélange réactionnel, on ajoute successivement de l'eau (2,98 ml) et de la soude 2N (2,98 ml). Après 10 minutes, on

rajoute de l'eau (2,98 ml). Le précipité formé est filtré sur Büchner et le filtrat est concentré à l'évaporateur rotatif pour donner le 15-octadécylamino-pentadécanol.

**<sup>1</sup>H RMN (CDCl<sub>3</sub>)** : δ (ppm) 0,88 (t, 3H, *J*= 6,96 Hz, H-33), 1,26 (m, 54H, (CH<sub>2</sub>)<sub>27</sub>), 1,43-1,59 (m, 7H, H-2, H-14, H-17 et bande NH), 1,5-1,6 (m, 4H, H-2 et H-13), 2,60 (t, 4H, *J*= 6,50 Hz, H-15 et H-16), 3,64 (t, 2H, *J*= 5,84 Hz, H-1).

**<sup>13</sup>C RMN (CDCl<sub>3</sub>)** : δ (ppm) 14,48 (C-33), 25,3 et 26,3 (C-2 et C-14), 29,72 ((CH<sub>2</sub>)<sub>27</sub>), 51,7 (C-15 et C-16), 63,6 (C-1).

*e) Synthèse du N-[benzyloxycarbonyl]-15-octadécylamino-pentadécanol*

On ajoute goutte à goutte 7,89 ml de chloroformate de benzyle (55,26 mmol) à une solution refroidie à 0°C de 15-octadécylamino-pentadécanol obtenu à l'étape précédente d) (13,71 g ; 27,63 mmol) et de triéthylamine (7,7 ml ; 55,26 mmol) dans du dichlorométhane sec (150 ml). Après 10 minutes, on vérifie le pH du mélange. Le mélange réactionnel est ensuite laissé à température ambiante pendant une nuit. Puis, la solution est lavée par de l'eau, séchée sur sulfate de magnésium (MgSO<sub>4</sub>) et concentrée. Le mélange réactionnel est purifié par chromatographie (heptane/AcOEt 6 : 4). On obtient le N-[benzyloxycarbonyl]-15-octadécylamino-pentadécanol avec un rendement de 70%.

**<sup>1</sup>H RMN (CDCl<sub>3</sub>)** : δ (ppm) 0,88 (t, 3H, *J*= 6,96 Hz, H-33), 1,26 (m, 54H, (CH<sub>2</sub>)<sub>27</sub>), 1,43-1,59 (m, 6H, H-2, H-14, H-17), 3,20-3,22 (m, 4H, H-15 et H-16), 3,64 (t, 2H, *J*= 5,84 Hz, H-1), 5,12 (s, 2H, OCH<sub>2</sub>Phe), 7,34 (m, 5H, Phe).

**<sup>13</sup>C RMN (CDCl<sub>3</sub>)** : δ (ppm) 14,48 (C-33), 25,8, 26,9 et 31,94 (C-2, C-14 et C-17), 29,72 ((CH<sub>2</sub>)<sub>27</sub>), 47,26-48,04 (C-15 et C-16), 63,08 (C-1), 66,79 (OCH<sub>2</sub>), 128,40 (Phe).

*f) Synthèse du 2,3,4-tri-O-acétyl-6-désoxy-β-L-galactopyrannoside du 15-[N-(benzyloxycarbonyl)-octadécylamino]-pentadécanyle*

1,5 g de fucose tétraacétylé (4,52 mmol) sont mis à réagir avec 0,634 ml de tétrachlorure d'étain (5,42 mmol) dans de l'acétonitrile séché (50 ml) pendant 30

minutes. Puis on ajoute 3,132 g de N-[benzyloxycarbonyl]-15-octadécylamino-pentadécanol obtenus à l'étape précédente e) (4,97 mmol). Après 5 heures, la réaction est extraite et le produit obtenu est alors purifié par chromatographie (heptane/acétate d'éthyle 6 : 4). Le rendement est de 69 %.

5 **<sup>1</sup>H RMN** :  $\delta$  (ppm) 0,87 (t, 3H,  $J = 6,96$  Hz, H-33), 1,2 (d, 3H,  $J = 6,51$  Hz, H-6), 1,25 (m, 54H,  $(\text{CH}_2)_{27}$ ), 1,52 (m, 6H,  $\text{OCH}_2\text{CH}_2$ , H-14 et H-17), 1,95, 2,05 et 2,15 (s, 3H,  $\text{OCOCH}_3$ ), 3,14-3,25 (m, 4H, H-15 et H-16), 3,44 (m, 1H,  $\text{OCH}_a\text{CH}_2$ ), 3,63 (m, 1H,  $\text{OCH}_b\text{CH}_2$ ), 3,79 (m, 1H, H-5), 4,41 (d, 1H,  $J = 7,98$  Hz, H-1), 4,99 (dd, 1H,  $J = 3,52$  Hz et  $10,46$  Hz, H-3), 5,09 (s, 2H,  $\text{OCH}_2\text{Phe}$ ), 5,16 (dd, 1H,  $J = 7,98$  Hz et  $10,46$  Hz, H-2), 5,23 (dd,  $J = 3,52$  Hz et  $3,31$  Hz, H-4), 7,32 (m, 5H, Phe).

**<sup>13</sup>C RMN ( $\text{CDCl}_3$ )** :  $\delta$  (ppm) 14,68 (C-33), 17,31 (C-2), 20,75 ( $\text{CH}_3\text{COO}$ ), 27,29 (C-6), 29,72 ( $(\text{CH}_2)_{27}$ ), 25,89-31,98 ( $\text{OCH}_2\text{CH}_2$ , C-14, C-17), 47,25-48,04 (C-15 et C-16), 66,91 ( $\text{CH}_2\text{Phe}$ ), 69,63 ( $\text{OCH}_2\text{CH}_2$ ), 69,45 (C-2), 70,57 (C-5), 70,85 (C-4), 71,44 (C-3), 96,25 (C-1), 128,43 (Phe), 156,21 et 171,30 (CO).

15 ***g) Synthèse du 2,3,4-tri-O-acétyl-6-désoxy- $\beta$ -L-galactopyrannoside du 15-octadécylamino-pentadécanyle***

A une solution de 2,3,4-tri-O-acétyl-6-désoxy- $\beta$ -L-galactopyrannoside du 15-[N-(benzyloxycarbonyl)-octadécylamino]-pentadécanyle obtenu à l'étape précédente f) (2,72 g ; 4,23 mmol) dans du méthanol (100 ml), on ajoute du palladium sur charbon  
20 actif à 10% (0,5 g) sous pression d'hydrogène. La réaction est quantitative.

**<sup>1</sup>H RMN** :  $\delta$  (ppm) 0,87 (t, 3H,  $J = 6,96$  Hz, H-33), 1,2 (d, 3H,  $J = 6,51$  Hz, H-6), 1,25 (m, 54H,  $(\text{CH}_2)_{27}$ ), 1,52 (m, 6H,  $\text{OCH}_2\text{CH}_2$ , H-14 et H-17), 1,88-1,93 (bande NH), 1,95, 2,05 et 2,15 (s, 3H,  $\text{OCOCH}_3$ ), 2,64 (m, 4H, H-15 et H-16), 3,46 (m, 1H,  $\text{OCH}_a\text{CH}_2$ ), 3,63 (m, 1H,  $\text{OCH}_b\text{CH}_2$ ), 3,79 (m, 1H, H-5), 4,41 (d, 1H,  $J = 7,98$  Hz, H-1), 4,99 (dd, 1H,  $J = 3,52$  Hz et  $10,46$  Hz, H-3), 5,16 (dd, 1H,  $J = 7,98$  Hz et  $10,46$  Hz, H-2), 5,23 (dd,  $J = 3,52$  Hz et  $3,31$  Hz, H-4).

**<sup>13</sup>C RMN ( $\text{CDCl}_3$ )** :  $\delta$  (ppm) 14,68 (C-33), 17,31 (C-2), 20,75 ( $\text{CH}_3\text{COO}$ ), 27,29 (C-6), 29,72 ( $(\text{CH}_2)_{27}$ ), 25,89-31,98 ( $\text{OCH}_2\text{CH}_2$ , C-14, C-17), 47,75-48,04 (C-15 et

C-16), 69,63 ( $\text{OCH}_2\text{CH}_2$ ), 69,45 (C-2), 70,57 (C-5), 70,85 (C-4), 71,44 (C-3), 96,25 (C-1), 171,30 (CO).

*h) Synthèse du 2,3,4-tri-O-acétyl-6-désoxy-β-L-galactopyrannoside du (3-[4-(3-amino-propyl-amino)-butyl-amino-propyl-benzyloxycarbonyl-amino]-méthylène - carbamoyl)-15-pentadécanyl-16-octadécanyle*

A une solution de 0,60 g du composé obtenu à l'étape précédente g) (0,94 mmol) dans du chloroforme (15 ml), on ajoute successivement de la diisopropyléthylamine (0,491 ml ; 2,82 mmol), du BOP (0,457 g ; 1,03 mmol) et de l'acide {3-[4-(3-benzyloxycarbonyl-amino-propyl-benzyloxycarbonyl-amino)-butyl-benzyloxycarbonyl-amino]-propylamino}-acétique obtenu à l'étape a) (0,748 g ; 0,94 mmol). L'huile résultante est purifiée par chromatographie (heptane/acétate d'éthyle 4 : 6). On obtient le 2,3,4-tri-O-acétyl-6-désoxy-β-L-galactopyrannoside du (3-[4-(3-amino-propyl-amino)-butyl-amino-propyl-benzyloxycarbonyl-amino]-méthylène-carbamoyl)-15-pentadécanyl-16-octadécanyle avec un rendement de 45 %.

<sup>1</sup>H RMN : δ (ppm) 0,87 (t, 3H,  $J = 6,96$  Hz, H-33), 1,2 (d, 3H,  $J = 6,51$  Hz, H-6), 1,24 (m, 54H,  $(\text{CH}_2)_{27}$ ), 1,39-1,67 (m, 15H,  $\text{OCH}_2\text{CH}_2$ , H-14, H-17, NH,  $\text{CH}_2$ ), 1,95, 2,05 et 2,15 (s, 3H,  $\text{OCOCH}_3$ ), 3,05-3,35 (m, 18H, H-15, H-16 et  $\text{CH}_2\text{N}$ ), 3,43 (m, 1H,  $\text{OCH}_a\text{CH}_2$ ), 3,67 (m, 1H,  $J = 6,74$  Hz,  $\text{OCH}_b\text{CH}_2$ ), 3,79 (m, 1H, H-5), 4,41 (d, 1H,  $J = 7,98$  Hz, H-1), 4,99 (dd, 1H,  $J = 3,52$  Hz et  $10,46$  Hz, H-3), 5,05 (s, 8H,  $\text{CH}_2\text{Phe}$ ), 5,16 (dd, 1H,  $J = 7,98$  Hz et  $10,46$  Hz, H-2), 5,23 (dd,  $J = 3,52$  Hz et  $3,31$  Hz, H-4), 5,47 (bande CONH, 1H), 7,32 (m, 20H, Phe).

<sup>13</sup>C RMN ( $\text{CDCl}_3$ ) : δ (ppm) 14,84 (C-33), 20,75 ( $\text{CH}_3\text{COO}$ ), 27,29 (C-6'), 29,72 ( $(\text{CH}_2)_{27}$ ), 25,89-31,98 ( $\text{OCH}_2\text{CH}_2$ , C-14, C-17 et  $\text{CH}_2$ ), 37,87-46,87 (C-15, C-16 et C-N), 66,84 ( $\text{CH}_2\text{Phe}$ ), 68,63 ( $\text{OCH}_2\text{CH}_2$ ), 69,45 (C-2), 70,57 (C-5), 70,85 (C-4), 71,44 (C-3), 96,25 (C-1), 128,31 (Phe), 157,01 et 171,30 (CO).

*i) Synthèse du 6-désoxy-β-L-galactopyrannoside du 1-[4-(3-amino-propyl-amino)-butyl-amino-propyl-benzyloxycarbonyl-amino]-méthylène -carbamoyl)-15-pentadécanyl-16-octadécanyle*

A une solution méthanolique (3 ml) contenant le produit obtenu à l'étape précédente h) (0,60 g ; 0,94 mmol) on ajoute une solution méthanolique (1 ml) saturée d'ammoniac. Après une heure, on concentre.

<sup>1</sup>H RMN :  $\delta$  (ppm) 0,87 (t, 3H,  $J=6,96$  Hz, H-33), 1,2 (d, 2H,  $J=6,51$  Hz, H-6), 1,24 (m, 54H, (CH<sub>2</sub>)<sub>27</sub>), 1,39-1,67 (m, 15H, OCH<sub>2</sub>CH<sub>2</sub>, H-14, H-17, NH, CH<sub>2</sub>), 3,05-3,35 (m, 18H, H-15, H-16 et CH<sub>2</sub>N), 3,4-3,7 (m, 6H, OCH<sub>2</sub>CH<sub>2</sub>, H-3, H-4, H-5, H-2), 4,73 (d, 1H,  $J=7,98$  Hz, H-1), 5,05 (s, 8H, CH<sub>2</sub>Phe), 5,47 (bande CONH, 1H), 7,32 (m, 20H, Phe).

j) *Synthèse du 6-désoxy- $\beta$ -L-galactopyrannoside du 1-[-(3-[4-(3-amino-propyl-amino)-butyl-amino-propyl-amino]-méthylène-carbamoyl)-15-pentadécanyl-16-octadécanyl (composé 3)*

A une solution du produit obtenu à l'étape précédente i) (0,072 g ; 0,05 mmol), on ajoute du palladium sur charbon à 10% (0,032 g) dans du méthanol. Après une nuit, on filtre sur papier en verre et on concentre à l'évaporateur rotatif. Le produit est ensuite purifié par CLHP sur une colonne préparative de type C-4.

<sup>1</sup>H RMN :  $\delta$  (ppm) 0,87 (t, 3H,  $J=6,96$  Hz, H-33), 1,2 (d, 2H,  $J=6,51$  Hz, H-6), 1,24 (m, 54H, (CH<sub>2</sub>)<sub>27</sub>), 1,39-1,67 (m, 15H, OCH<sub>2</sub>CH<sub>2</sub>, H-14, H-17, CH<sub>2</sub>), 2,92-3,19 (m, 18H, H-15, H-16 et CH<sub>2</sub>N), 3,4-3,7 (m, 6H, OCH<sub>2</sub>CH<sub>2</sub>, H-3, H-4, H-5, H-2), 4,73 (d, 1H,  $J=7,98$  Hz, H-1).

## 20 C \ UTILISATION DES AGENTS DE TRANSFERT SELON L'INVENTION

### Exemple 4 : préparation de complexes agent de transfert/acide nucléique avec le composé 2 et mesure de leur taille

Cet exemple illustre la préparation de complexes entre un agent de transfert selon l'invention et un acide nucléique, leur taille ayant ensuite été mesurée.

25 Le glycolipide utilisé dans cet exemple et dans les exemples qui suivent est le composé 2, en solution dans du chloroforme, à une concentration de 10 mg/ml. Dans

certain cas, un co-lipide neutre, cholestérol ou DOPE, a été préalablement mélangé au composé 2.

La solution lipidique est préparée de la façon suivante : un échantillon de quantité désirée est prélevé, le solvant est évaporé sous flux d'argon et on laisse sécher pendant 1 heure. Puis, le lipide est réhydraté avec une solution contenant du dextrose 5% et 10 mM de chlorure de sodium pendant toute une nuit à 4°C. Le jour suivant, les solutions lipidiques sont chauffées à 60°C pendant 5 minutes puis passées aux ultrasons pendant 1 minute. L'opération est répétée jusqu'à ce que la taille des particules lipidiques soit stable.

L'ADN utilisé est le plasmide pXL3031 (figure 1) en solution dans un mélange de dextrose 5% et de chlorure de sodium 10 mM à une concentration de 0,5 mg/ml ou de 1,0 mg/ml. Ce plasmide contient le gène luc codant pour la luciférase sous contrôle du promoteur P/E CMV du cytomégalo virus. Sa taille est de 3671 bp. Le schéma de ce plasmide est représentée à la figure 1. Le plasmide pXL3031 a été purifié selon les méthodes décrites dans la demande de brevet WO 97/35002.

Les complexes composé 2/ADN sont préparés en mélangeant rapidement des volumes appropriés de solution d'ADN plasmidique et de composé 2 (selon le rapport de charges désiré), à température ambiante. La quantité d'agent transfectant varie entre 0,25 nmoles/ $\mu$ g d'ADN et 12 nmoles/ $\mu$ g d'ADN.

La taille des complexes a été analysée en mesurant le diamètre hydrodynamique par diffusion dynamique de la lumière (Dynamic Laser Light Scattering) à l'aide d'un appareil Coulter N4Plus. Les échantillons sont dilués 20 fois dans une solution contenant 5% de dextrose et 20 mM de chlorure de sodium pour éviter les diffusions multiples.

A un rapport de 3 nmoles de lipide/ $\mu$ g d'ADN, les résultats suivants ont été obtenus :

	Taille en nm
Micelles	130 nm
formulation avec du Cholestérol	153 nm
Formulation avec de la DOPE	137 nm



Le terme "micelles" indique que le composé 2 a été utilisé seul, c'est-à-dire sans ajout de co-lipide neutre, et il forme donc une solution micellaire.

Ce tableau montre que les complexes obtenus ont une taille comprise entre 130 nm et 150 nm environ, ce qui est compatible avec une utilisation pharmaceutique, notamment en injection.

**Exemple 5 : Comportement des complexes formés à partir du composé 2 à différents rapports de charge**

Cet exemple illustre le comportement des complexes agent de transfert selon l'invention/acide nucléique lorsqu'on fait varier le rapport de charge. L'impact de l'ajout d'un co-lipide (cholestérol ou DOPE) est également illustré.

De façon classique, on distingue 3 phases physico-chimiques lorsqu'on augmente le rapport de charges agents de transfert/ADN (B. Pitard et al., *Virus-sized self-assembling lamellar complexes between plasmid DNA and cationic micelles promote gene transfer*, PNAS, Vol. 94, pp. 14412-14417, 1997). Ces trois phases déterminent le potentiel thérapeutique de l'agent de transfert.

A faible rapport de charge, l'ADN n'est pas saturé par l'agent de transfert. Il reste encore de l'ADN non-complexé, et les complexes sont globalement chargés négativement et de petite taille. Cette phase, stable, est appelée "Phase A".

Le fait que l'ADN ne soit pas complètement saturé par l'agent de transfert signifie que l'ADN n'est pas complètement protégé. L'ADN peut donc être soumis aux dégradations par les nucléases. Par ailleurs, les complexes étant globalement négatifs, le passage de la membrane cellulaires est difficile. Pour ces raisons, les complexes nucléolipidiques de la phase A sont relativement inactifs.

A rapport de charge intermédiaire, l'ADN est complètement saturé par l'agent de transfert, et les complexes sont globalement neutres ou légèrement positifs. Cette phase est instable car les répulsions ioniques sont minimales et un phénomène de d'agrégation peut se produire. La taille des particules est bien au dessus de la limite

de détection par diffusion dynamique de la lumière (très supérieure à 3  $\mu\text{m}$ ). Cette phase instable est appelée "phase B". Une telle taille de complexes n'est pas adaptée pour des utilisations en injection, bien que cela ne signifie pas que les complexes soient inactifs dans la phase B : ils sont seulement sous une formulation qui n'est pas appropriée pour leur injection dans un but pharmaceutique.

A rapport de charge plus élevé, l'ADN est sursaturé par l'agent de transfert, et les complexes sont globalement positifs. Du fait des fortes répulsions entre les charges positives, cette phase est stable. Elle est désignée sous le nom de "phase C". Contrairement à la phase A, les complexes obtenus sont sous une forme telle que l'ADN est très bien protégé vis-à-vis des nucléases, et la charge globalement positive de ces complexes facilite la fixation sur la membrane cellulaire de nature anionique et le passage de cette membrane. Les complexes de la phase C sont donc particulièrement adaptés à une utilisation pour le transfert d'acides nucléiques dans les cellules.

Ces 3 zones A, B et C ont été également mises à jour avec le composé 2 selon l'invention comme agent de transfert :

Rapport de charges	0,25	0,5	0,75	1	1,5	2	3	4	6	8	10	12
Micelles	A	A	B	B	B	B	C	C	C	C	C	C
+ Cholestérol	A	A	B	B	B	C	C	C	C	C	C	C
+ DOPE	A	A	B	B	B	C	C	C	C	C	C	C

Comme le montre le tableau ci-dessus, la zone B, qui est la zone d'instabilité, est particulièrement petite et se situe à des rapports de charge très faibles. La zone C commence dès 2 nmoles de lipide/ $\mu\text{g}$  d'ADN lorsque le composé 2 est utilisé conjointement à un co-lipide (Cholestérol ou DOPE), et à partir de 3 nmoles de lipide/ $\mu\text{g}$  d'ADN lorsque le composé est utilisé seul. Comme cela a été précisé précédemment, c'est dans cette zone qu'il est particulièrement avantageux de se placer pour une utilisation pharmaceutique.

A titre de comparaison, il a été montré avec un des lipides cationiques divulgué dans la demande WO 97/18185 que la zone C commençait à se former à des rapports de charge au moins égal à 2 selon la concentration en chlorure de sodium de la solution (voir figure 3A dans B. Pitard et al., PNAS USA, 94, pp. 14412-14417, 1997).

- 5    Ainsi, le composé 2 est un agent de transfert particulièrement avantageux car il est stable à de faibles rapports de charge, ce qui permet de former des complexes stables avec de faibles quantités de glycolipides, avec les conséquences bénéfiques qui en découle sur le plan de la toxicité.

**Exemple 6 : utilisation du composé 2 pour le transfert *in vitro* d'ADN**

- 10   Cet exemple illustre la capacité des agents de transfert selon l'invention à transfecter l'ADN dans les cellules *in vitro*, à différents rapports de charge, en l'absence et en présence d'un co-lipide neutre (cholestérol ou DOPE).

- 15   Des microplaques de 24 puits sontensemencées avec 60000 cellules HeLa par puit, et sont mises en croissance une nuit. Le nombre de cellules après une nuit, est donc au moment de la transfection, est de 100000 cellules par puit.

- Chaque puit est mis en contact avec les complexes formés avec le composé 2 et contenant 1 µg d'ADN plasmidique dans 0,5 ml de milieu de culture DMEM (Gibco/BRL sans sérum.. Les cellules sont incubées à 37°C pendant 5 heures. Le milieu contenant les complexes est ensuite enlevé et remplacé par un milieu de culture DMEM et 10% de sérum de veau foetal. Puis, les cellules sont à nouveau mises en culture pendant 24 heures. Enfin, les cellules sont lysées et testées en utilisant un kit de test de luciférase (Promega) et un luminomètre Dynex MLX.
- 20

- Les résultats obtenus sont indiqués sur l'histogramme de la figure 2. L'efficacité du transfert est représentée par l'expression de la luciférase en pg/puit. On constate que la transfection maximale est de 500 pg/puit environ.
- 25

En conclusion, cet exemple montre clairement qu'il est possible d'utiliser le composé 2 selon l'invention pour former des complexes susceptible de promouvoir le transfert de l'ADN dans les cellules *in vitro*.

**Exemple 7 : utilisation du composé 2 pour le transfert *in vivo* d'ADN**

- 5 Cet exemple illustre la capacité des agents de transfert selon l'invention à transférer l'ADN dans les cellules *in vivo*.

Le transfert de gène *in vivo* a été effectué sur des souris Balb/C par administration intratrachéale, intraveineuse et intramusculaire.

- 10 Dans le cas des injections intramusculaires, chaque souris a reçu 30 µl de formulation contenant 15 µg d'ADN plasmidique dans le muscle antérieur du tibia. Les tissus sont récupérés 7 jours après l'injection, ils sont congelés et stockés à -80°C en attendant d'effectuer les tests d'activité luciférase.

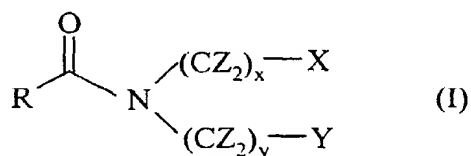
- 15 Dans le cas des injections par voie intraveineuse, chaque souris a reçu 200 µl de formulation contenant 50 µg d'ADN plasmidique. Les tissus sont récupérés 24 heures après l'injection, puis sont congelés et stockés de la même façon que précédemment.

La figure 3 illustre l'activité des complexes formés avec le composé 2 pour le transfert de gène *in vivo* en intramusculaire. Ces résultats montrent clairement que la formation de complexes avec le composé 2 selon l'invention et de l'ADN permet de promouvoir le transfert dudit ADN dans les cellules *in vivo*.

- 20 De la même façon, on peut utiliser tout agent de transfert tel que défini dans la présente invention pour promouvoir le transfert d'ADN dans les cellules de tout type de tissus.

## REVENDICATIONS

1. Agents de transfert d'acides nucléiques caractérisés en ce qu'ils comprennent un espaceur hydrophobe lié chimiquement d'une part à un polycation et d'autre part à au moins un substituant hydrophile.
- 5 2. Agents de transfert d'acides nucléiques selon la revendication 1 caractérisés en ce que ledit espaceur hydrophobe est constitué de 2 ou 3 chaînes grasses linéaires hydrocarbonées comprenant entre 10 et 20 atomes de carbone par chaîne, chaque chaîne pouvant être de longueur différente, ou bien ledit espaceur hydrophobe est constitué d'une très longue chaîne grasse linéaire hydrocarbonée, comprenant entre 20  
10 et 50 atomes de carbone.
3. Agents de transfert d'acides nucléiques selon la revendication 1 caractérisés en ce que le ou les substituants hydrophiles sont choisis parmi les substituants hydroxy, amino, les polyols, les sucres, ou encore les peptides hydrophiles.
4. Agents de transfert d'acides nucléiques selon la revendication 1 ou 3 caractérisés en  
15 ce que l'un au moins des substituants hydrophiles est un sucre.
5. Agents de transfert d'acides nucléiques selon la revendication 1 de formule générale (I) :



pour laquelle :

- 20 - R représente un polycation,
- Z représente un atome d'hydrogène ou un atome de fluor, les différents Z étant indépendant les uns des autres, et
- soit x et y, indépendamment l'un de l'autre, représentent des entiers compris entre 10 et 22 inclus, et X et Y, indépendamment l'un de l'autre, représentent un atome

d'hydrogène, un groupement -OAlk où Alk représente un alkyle droit ou ramifié contenant 1 à 4 atomes de carbone, un groupe hydroxy, un groupement amino, un polyol, un sucre, un peptide hydrophile ou non-hydrophile, ou un oligonucléotide,

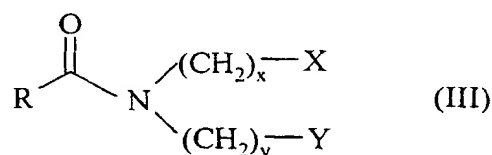
étant entendu que l'un au moins des substituants X et Y représente un groupe hydrophile choisi parmi les hydroxy, les amino, les polyols, les sucres, ou les peptides hydrophiles,

- soit x est égal à 0 ou 1, y est un entier compris entre 20 et 50, X est soit un atome d'hydrogène soit un groupement -OAlk où Alk représente un alkyle droit ou ramifié contenant 1 à 4 atomes de carbone, et Y est un groupe hydrophile choisi parmi les

hydroxy, les amino, les polyols, les sucres, ou les peptides hydrophiles,

le cas échéant sous leurs formes isomères, ainsi que leurs mélanges, ou leurs sels lorsqu'ils existent.

6. Agents de transfert d'acides nucléiques selon la revendication 1 ou 5 de formule générale (III) :



15

pour laquelle :

- R représente un polycation, et

- soit x et y, indépendamment l'un de l'autre, représentent des entiers compris entre 10 et 22 inclus, et X et Y, indépendamment l'un de l'autre, représentent un atome

d'hydrogène ou un sucre, étant entendu que l'un au moins des substituants X et Y représente un sucre,

- soit x est égal à 0 ou 1, y est un entier compris entre 20 et 50, X est un atome d'hydrogène et Y est un sucre.

le cas échéant sous leurs formes isomères, ainsi que leurs mélanges, ou leurs sels

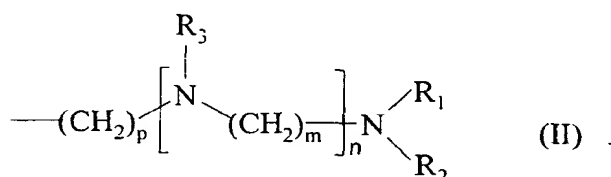
lorsqu'ils existent.

25

7. Agents de transfert d'acides nucléiques selon la revendication 6 caractérisés en ce que x et y, indépendamment l'un de l'autre, représentent des entiers compris entre 10 et 22 inclus, et l'un de X et Y représente un atome d'hydrogène et l'autre un sucre.

8. Agents de transfert d'acides nucléiques selon l'une des revendications 1 et 5 à 7 caractérisés en ce que ledit polycation est une polyamine linéaire ou ramifiée, chaque groupe amino étant séparé par un ou plusieurs groupes méthylène.

9. Agents de transfert d'acides nucléiques selon la revendication 8 caractérisés en ce que ledit polycation a pour formule générale (II) :



10 dans laquelle :

-  $\text{R}_1$ ,  $\text{R}_2$  et  $\text{R}_3$  représentent indépendamment les uns des autres un atome d'hydrogène ou un groupement  $(\text{CH}_2)_q\text{NR}'\text{R}''$  avec q un nombre entier pouvant varier de 1 à 6, ceci de manière indépendante entre les différents groupements  $\text{R}_1$ ,  $\text{R}_2$  et  $\text{R}_3$ , étant entendu que l'un au moins de  $\text{R}_1$ ,  $\text{R}_2$  et  $\text{R}_3$  est différent d'un atome d'hydrogène,

15 -  $\text{R}'$  et  $\text{R}''$  représentent indépendamment l'un de l'autre un atome d'hydrogène ou un groupement  $(\text{CH}_2)_q\text{NH}_2$  avec q défini comme précédemment,

- m représente un nombre entier compris entre 1 et 6, et

- n et p représentent indépendamment l'un de l'autre des nombres entiers compris entre 0 et 6, avec lorsque n est supérieur ou égal à 2, m pouvant prendre des valeurs

20 différentes et  $\text{R}_3$  des significations différentes au sein de la formule générale (II), et lorsque n est égal à 0, l'un au moins des substituants  $\text{R}_1$  et  $\text{R}_2$  est différent d'un atome d'hydrogène.

10. Agents de transfert d'acides nucléiques selon l'une des revendications 1 et 5 à 7 caractérisés en ce que ledit polycation est choisi parmi la spermine, la spermidine, la

cadavérine, la putrescine, l'hexaméthylènetétramine (hexamine), le chlorure de méthacrylamidopropyl-triméthylammonium (AMBTAC), le chlorure de 3-acrylamido-3-méthylbutyltriméthylammonium (AMBTAC), les polyvinylamines, les polyéthylèneimines, ou les ionènes.

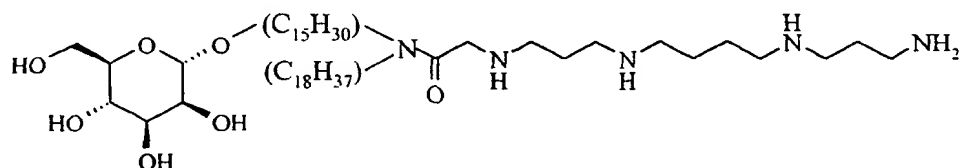
5 11. Agents de transfert d'acides nucléiques selon l'une des revendications 3 à 7 caractérisés en ce que le ou les sucres sont des molécules de mono-, oligo- ou polysaccharide.

12. Agents de transfert d'acides nucléiques selon la revendication 11 caractérisés en ce que ledit ou lesdits sucres sont choisis parmi le glucose, le mannose, le rhamnose,  
10 le galactose, le fructose, le maltose, le lactose, le saccharose, le sucrose, le fucose, le cellobiose, l'allose, le laminarabiose, le gentiobiose, le sophorose, le mélibiose, le dextran, l' $\alpha$ -amylose, l'amylopectine, les fructans, les mannans, les xylans et les arabinans.

13. Agents de transfert d'acides nucléiques selon la revendication 5 caractérisés en ce  
15 que ledit oligonucléotide est toute chaîne contenant un ou plusieurs nucléotides, désoxynucléotides, ribonucléotides et /ou désoxyribonucléotides, éventuellement couplée à une ou plusieurs molécules ayant des propriétés distinctes.

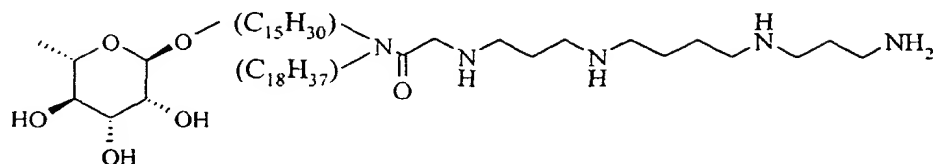
14. Agents de transfert d'acides nucléiques selon la revendication 5 caractérisés en ce  
20 que ledit peptide est toute chaîne contenant un ou plusieurs acides aminés liés entre eux par des liaisons de nature peptidique, éventuellement substituée par un ou plusieurs groupes aliphatiques qui peuvent être saturés ou insaturés, et linéaires, ramifiés ou cycliques.

15. Agent de transfert selon la revendication 1 de formule :

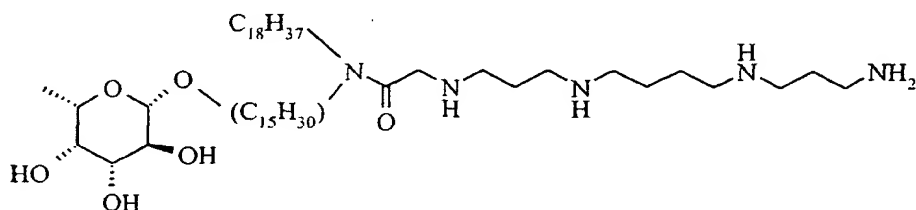




16. Agent de transfert selon la revendication 1 de formule :



17 Agent de transfert selon la revendication 1 de formule :



- 5 18. Composition caractérisée en ce qu'elle contient un agent de transfert d'acides nucléiques tel que défini dans les revendications 1 à 17 et un acide nucléique.
19. Composition selon la revendication 18 caractérisée en ce que l'acide nucléique est un acide désoxyribonucléique ou bien un acide ribonucléique.
20. Composition selon la revendication 18 ou 19 caractérisée en ce que ledit acide
- 10 nucléique comprend un ou plusieurs gènes d'intérêt thérapeutique sous contrôle de séquences de régulation.
21. Composition selon les revendications 18 à 20 caractérisée en ce que ledit acide nucléique est un gène ou une séquence antisens.
22. Composition selon la revendication 18 caractérisée en ce qu'elle contient en outre
- 15 un ou plusieurs adjuvants.
23. Composition selon la revendication 22 caractérisée en ce que l'adjuvant est un ou plusieurs lipides neutres.
24. Composition selon la revendication 23 caractérisée en ce que les lipides neutres sont des lipides à deux chaînes grasses.

25. Composition selon les revendications 23 et 24 caractérisée en ce que les lipides neutres sont des lipides naturels ou synthétiques, zwitterioniques ou dépourvus de charge ionique dans les conditions physiologiques, choisis par exemple parmi la dioléoylphosphatidyléthanolamine (DOPE),  
5 l'oléylpalmitoylphosphatidyléthanolamine (POPE), les di-stéaroyl, -palmitoyl, -mirystoylphosphatidyléthanolamines ainsi que leurs dérivés N-méthylés 1 à 3 fois, les phosphatidylglycérols, les diacylglycérols, les glycosyldiacylglycérols, les cérebrosides (tels que notamment les galactocérebrosides), les sphingolipides (tels que notamment les sphingomyélines) ou encore les asialogangliosides (tels que  
10 notamment les asialoGM1 et GM2).
26. Composition selon la revendication 22 caractérisée en ce que ledit adjuvant est un composé intervenant directement ou non au niveau de la condensation de l'acide nucléique.
27. Composition selon la revendication 26 caractérisée en ce que ledit adjuvant dérive  
15 en tout ou en partie d'une protamine, d'une histone, ou d'une nucléoline et/ou de l'un de leur dérivés, ou bien est constitué, en tout ou en partie, de motifs peptidiques (KTPKKAKKP) et/ou(ATPAKKAA), le nombre des motifs pouvant varier entre 2 et 10, et pouvant être répétés de manière continue ou non.
28. Composition selon les revendications 18 à 27 caractérisée en ce qu'elle comprend  
20 un véhicule pharmaceutiquement acceptable pour une formulation injectable.
29. Composition selon les revendications 18 à 27 caractérisée en ce qu'elle comprend un véhicule pharmaceutiquement acceptable pour une application sur la peau et/ou les muqueuses.
30. Utilisation d'un agent de transfert tel que défini dans les revendications 1 à 17  
25 pour la fabrication d'un médicament destiné à traiter les maladies.

31. Méthode de traitement du corps humain ou animal comprenant les étapes suivantes :

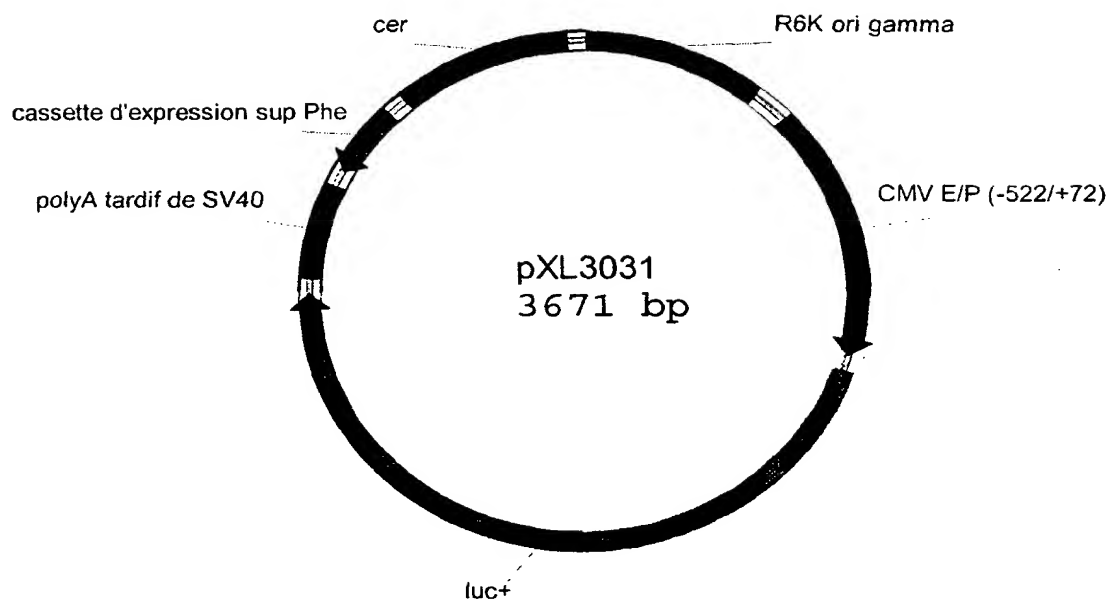
- (1) la mise en contact de l'acide nucléique avec un agent de transfert tel que défini dans les revendications 1 à 17, pour former un complexe, et
- 5 (2) la mise en contact des cellules du corps humain ou animal avec le complexe formé en (1).

32. Méthode de transfert d'acides nucléiques dans les cellules caractérisée en ce qu'elle comprend les étapes suivantes :

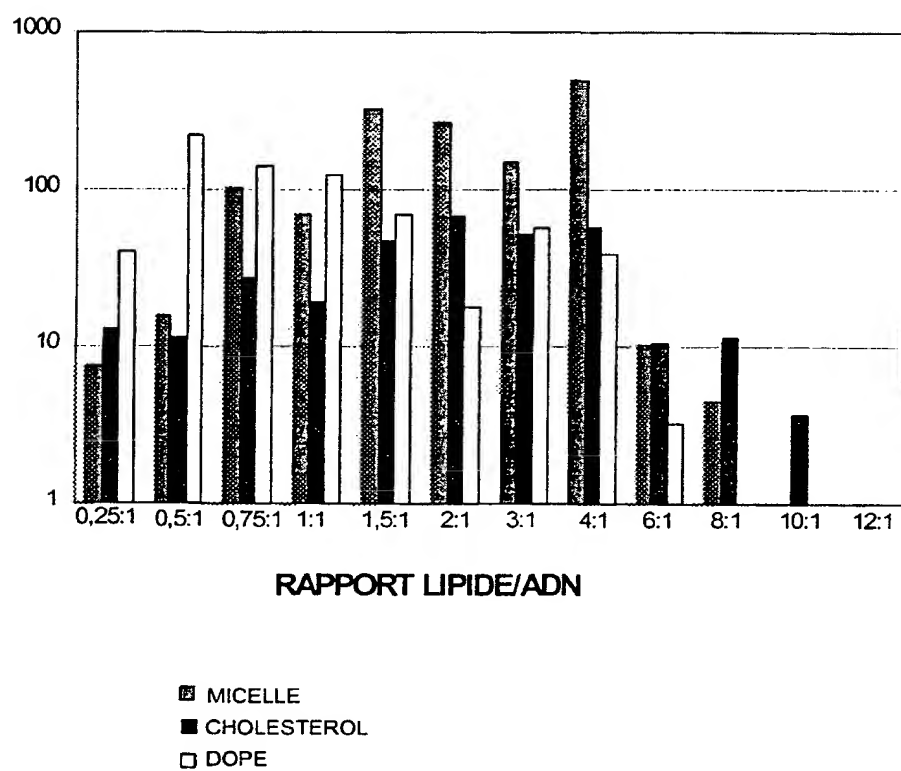
- (1) la mise en contact de l'acide nucléique avec un agent de transfert tel que défini, 10 pour former un complexe, et
- (2) la mise en contact des cellules avec le complexe formé en (1).

33. Méthode de transfert d'acides nucléiques dans les cellules selon les revendications 31 ou 32 caractérisée en ce que ledit agent de transfert et/ou ledit acide nucléique sont préalablement mélangés à un ou plusieurs adjuvant(s) tels que définis dans les 15 revendications 22 à 27.



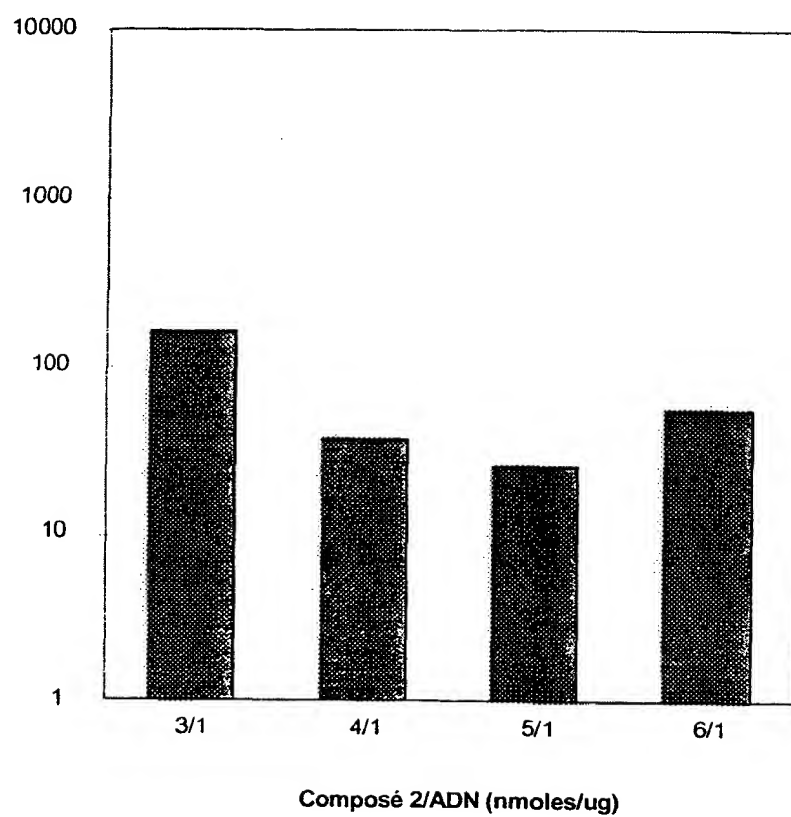
**FIG. 1**



**FIG. 2**





**FIG. 3****Luciférase (pg/muscle)**



## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

<b>(51) International patent classification<sup>7</sup>:</b>  C12N 15/87, A61K 48/00, C07H 15/04	A2	<b>(11) International publication number:</b> WO 00/32803  <b>(43) International publication date:</b> 8 June 2000 (08.06.00)
<b>(21) International application number:</b> PCT/FR99/02995  <b>(22) International filing date:</b> 2 December 1999 (02.12.99)  <b>(30) Data relating to the priority:</b> 98/15,309 3 December 1998 (03.12.98) FR 60/117,184 26 January 1999 (26.01.99) US  <b>(71) Applicant (for all designated States except US):</b> AVENTIS PHARMA S.A. [FR/FR]; 20 avenue Raymond Aron, F-92160 Antony (FR).  <b>(72) Inventors; and</b> <b>(75) Inventors/Applicants (US only):</b> HERSCOVICI, Jean [FR/FR]; 14 rue du Château des Rentiers, F-75013 Paris (FR). HOFLAND, Hans [NL/US]; 1439 Terra Nova Boulevard, Pacifica, CA 94044 (US). JACOPIN, Christophe [FR/FR]; 11 rue du Bois des Roches, F-91700 Sainte Geneviève des Bois (FR). SCHERMAN, Daniel [FR/FR]; 10 rue Erard, F-75012 Paris (FR).  <b>(74) Representative:</b> LAURENT, Claire; Aventis Pharma, Direction Brevets Tri LE1 144, 20 avenue Raymond Aron, F-92165 Antony Cedex (FR).		<b>(81) Designated states:</b> AE, AL, AU, BA, BB, BG, BR, CA, CN, CR, CU, CZ, DM, GD, GE, HR, HU, ID, IL, IN, IS, JP, KP, KR, LC, LK, LR, LT, MA, MG, MK, MN, MX, NO, NZ, PL, RO, RU, SG, SI, SK, SL, TR, TT, TZ, UA, US, UZ, VN, YU, ZA, ARIPO Patent (GH, GM, KE, LS, MW, SD, SL, SZ, TZ, UG, ZW), Eurasian Patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European Patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI Patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).  <b>Published</b> <i>Without the International Search Report and to be  republished once the report has been received.</i>
As printed		
<b>(54) Title:</b> NOVEL NUCLEIC ACID TRANSFERRING AGENTS, COMPOSITIONS CONTAINING THEM AND USES  <b>(54) Titre:</b> NOUVEAUX AGENTS DE TRANSFERT D'ACIDES NUCLEIQUES, COMPOSITIONS LES CONTENANT ET LEURS UTILISATIONS  <b>(57) Abstract</b>  The invention concerns novel transfer agents, compositions containing them and their uses for transferring <i>in vitro</i> , <i>in vivo</i> or <i>ex vivo</i> nucleic acids into cells. More precisely, the invention concerns novel nucleic acid transfer agents comprising a hydrophobic spacer chemically bound to a polycation and to at least a hydrophilic substituent.  <b>(57) Abrégé</b>  La présente invention se rapporte à de nouveaux agents de transfert, les compositions les contenant et leurs utilisations pour le transfert <i>in vitro</i> , <i>in vivo</i> ou <i>ex vivo</i> d'acides nucléiques dans les cellules. Plus précisément, la présente invention concerne des nouveaux agents de transfert d'acides nucléiques qui comprennent un espaceur hydrophobe lié chimiquement d'une part à un polycation et d'autre part à au moins un substituant hydrophile.		



# *ONLY FOR INFORMATION*

Codes used to identify the PCT member States on the flyleaves of the brochures in which international applications made under the PCT are published.

AL	Albania	ES	Spain	LS	Lesotho	SI	Slovenia
AM	Armenia	FI	Finland	LT	Lithuania	SK	Slovakia
AT	Austria	FR	France	LU	Luxembourg	SN	Senegal
AU	Australia	GA	Gabon	LV	Latvia	SZ	Swaziland
AZ	Azerbaijan	GB	United Kingdom	MC	Monaco	TD	Chad
BA	Bosnia-Herzegovina	GE	Georgia	MD	Republic of Moldova	TG	Togo
BB	Barbados	GH	Ghana	MG	Madagascar	TJ	Tajikistan
BE	Belgium	GN	Guinea	MK	Former Yugoslav Republic of Macedonia	TM	Turkmenistan
BF	Burkina Faso	GR	Greece	ML	Mali	TR	Turkey
BG	Bulgaria	HU	Hungary	MN	Mongolia	TT	Trinidad and Tobago
BJ	Benin	IE	Ireland	MR	Mauritania	UA	Ukraine
BR	Brazil	IL	Israel	MW	Malawi	UG	Uganda
BY	Belarus	IS	Iceland	MX	Mexico	US	United States of America
CA	Canada	IT	Italy	NE	Niger	UZ	Uzbekistan
CF	Central African Republic	JP	Japan	NL	Netherlands	VN	Vietnam
CG	Congo	KE	Kenya	NO	Norway	YU	Yugoslavia
CH	Switzerland	KG	Kyrgyzstan	NZ	New Zealand	ZW	Zimbabwe
CI	Ivory Coast	KP	Democratic People's Republic of Korea	PL	Poland		
CM	Cameroon	KR	Republic of Korea	PT	Portugal		
CN	China	KZ	Kazakhstan	RO	Romania		
CU	Cuba	LC	Saint Lucia	RU	Russian Federation		
CZ	Czech Republic	LI	Liechtenstein	SD	Sudan		
DE	Germany	LK	Sri Lanka	SE	Sweden		
DK	Denmark	LR	Liberia	SG	Singapore		
EE	Estonia						

